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THE HYDROLYSIS OF ADENOSINE TRIPHOSPHATE BY BRAIN SUSPENSIONS[1]

BY HANNA M. PAPPIUS AND K. A. C. ELLIOTT

Abstract

Adenosine triphosphate (ATP) hydrolysis by unfractionated brain suspensions has been measured manometrically and chemically in bicarbonate buffer. Both magnesium and, less strongly, calcium accelerate ATP hydrolysis by brain suspensions. The effectiveness of low magnesium or calcium concentrations is decreased by relatively high ATP concentrations. The optimum pH for phosphate liberation is about 8. The ratio of carbon dioxide evolved to inorganic phosphate liberated depends on the pH. Evidence is given that the suspensions contain a Mg-activated ATPase which is strongly inhibited by calcium, a Ca-activated ATPase, and a Mg-activated PPase which is strongly inhibited by calcium but is different from the Mg-ATPase. These enzymes were not obtained in "latent" form nor activated by glutathione. Ethylenediamine tetraacetate can inhibit Ca-ATPase completely but is less effective on the Mg-ATPase and can release the latter from calcium inhibition. Other inhibitors have been tested.

The ATPase activity of cerebral cortex of mouse, rat, cat, beef, and man has been measured by a standard procedure. The activity per unit weight decreases with increasing size of the animal parallel to the decrease in the oxygen uptake rate. Calculations show that the ATPase activity is high enough to release all high energy phosphate which could be produced by esterification coupled with respiration.

Introduction

The rate of acetylcholine synthesis by brain preparations (18, 20), and presumably the rates of other reactions which involve adenosine triphosphate (ATP), are dependent upon the concentration of ATP. Factors which determine the concentration of ATP in brain tissue are therefore of obvious physiological interest. The ATP concentration is stabilized to some extent by the reserve of high energy phosphate in creatine phosphate but this factor is possibly less significant in brain, where the creatine phosphate content is low, than in muscle where it is high. The ATP concentration would be determined largely by the rate of production of ATP and the rate of its breakdown. The production of high energy phosphate by respiring or glycolyzing tissue preparations has been studied by various workers (see, for

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Contribution from the Donner Laboratory of Experimental Neurochemistry, Montreal, Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University Montreal, Quebec, Canada. This work was reported in part to the XIX International Physiological Congress, Montreal, September 1953.

example (17)). Inorganic phosphate can be released from ATP by the action of specific ATPases, which release the terminal phosphate group, by specific ATPases with adenylate kinase (myokinase), which together will release both labile phosphate groups, probably by other phosphatases, and indirectly by transfer of the terminal phosphate group to an acceptor substance which is then hydrolyzed by a phosphatase. For convenience the term "ATPase" will be used for all systems which cause release of inorganic phosphate from ATP.

The literature on ATP hydrolysis is already large and often confusing; most of it is concerned with isolated enzymes. Dubois and Potter (5) described a method for determining total ATPase activity, but little further work on total activity has been reported. The present article describes observations made while developing a simple standard method for determining ATP-splitting activity in unfractionated brain tissue and results of application of the method. No attempt has been made to define the enzymes involved by isolation but observations have been made which indicate that more than one enzyme is active in brain. This study was undertaken primarily to establish, by interpolation or extrapolation from results obtained with various species, a probable average value and the range of variability of the activity of normal human cortex for comparison with the activity in abnormal human material. The variation of hydrolytic activity with species and a correlation with respiratory activity are discussed.

Methods and Materials

The adenylic acid derivatives were obtained from commercial sources and stored at low temperature. For most experiments the chromatographically purified sodium salt of ATP, as purchased, was used but many points were confirmed with solutions of the sodium salt prepared from the barium salt.

Whole rat cerebral hemispheres were used for the general studies. Pure gray cerebral cortex was used for the interspecies comparisons. The weighed fresh tissue was homogenized, by means of the Potter-Elvehjem apparatus, usually in 0.025 *M* sodium bicarbonate solution which had been treated with 5% carbon dioxide. A homogenizer of stainless steel or with a teflon plunger was used. Manometric studies were made with standard Warburg equipment. The gas phase, except where specified, was commercially obtained nitrogen – 5% carbon dioxide mixture which was freed from oxygen by passage over hot copper fragments. Inorganic and seven minute hydrolyzable phosphate was determined by the Fiske-Subbarow method as given by Umbreit *et al.* (25).

Experimental

The hydrolysis of ATP has been followed manometrically by measuring the carbon dioxide evolved from bicarbonate buffered medium by acid groups produced. This method is rapid and convenient for following the course of the reactions but, as will be discussed below, the results must be interpreted

with caution.* All main points have been checked by colorimetric phosphate determinations.

When solutions of magnesium or calcium salts are tipped into an ATP solution, both solutions being bicarbonate-buffered at the same pH, an immediate evolution of carbon dioxide occurs and is completed within five minutes. It is evident that these ions form complexes with ATP with resultant increase in dissociation of acid groups. Under the conditions of these experiments and when the ATP concentration exceeds that of the cation, about 0.8 equivalents of acid are produced per atom of magnesium and about 0.45 per atom of calcium. With inorganic pyrophosphate the extra ionization is greater, about 2.4 with magnesium and 1.3 with calcium. With inorganic phosphate the effect of magnesium is smaller than with ATP, about 0.25 equivalent; the effect of calcium increases greatly with increasing calcium concentration (see below). In the standard manometric procedure, the catalytic cation is mixed with the ATP solution and the tissue suspension is tipped in after equilibration.

Fig. 1 shows the typical course of carbon dioxide evolution. With a relatively high concentration of tissue, evolution of carbon dioxide is rapid until an amount nearly equivalent to two moles per mole of ATP, corrected for the CO_2/P ratio (see below), has been released. The evolution then falls

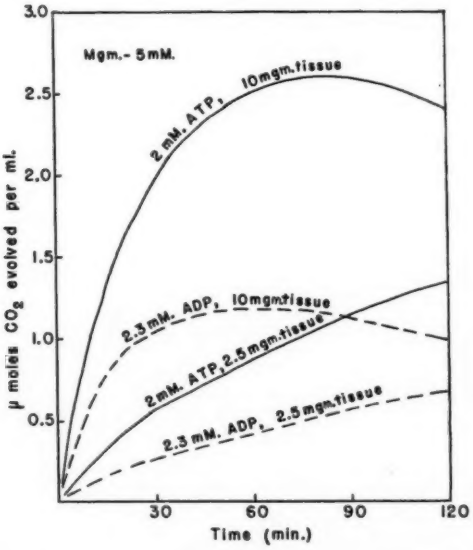

FIG. 1. Manometric observation of hydrolysis of ATP and ADP. The fresh weight of whole brain tissue is shown in mgm. per ml. of medium.

** Since much of this work was completed Green and Mommaerts (12) have published a titrimetric method for determining ATP splitting activity. This, like the manometric method, depends on the release of acid groups and in general would be subject to the same limitations.*

off completely and a slow uptake of carbon dioxide sets in. This uptake is the result of hydrolysis of adenosine monophosphate (AMP). (At the pH of the experiment the total ionization of inorganic phosphate is less than that of AMP (1).) With a low concentration of tissue, an initial rapid phase and a nearly steady second phase are observed. These two phases are apparent under all conditions, with ADP as well as with ATP. Obviously some change in the reaction occurs after the first few minutes but the nature of this change has not been discovered.

In the standard method adopted, the rate for the first 15 min., with low concentrations of tissue, is measured. With low tissue concentrations the initial rates per unit weight of tissue were found to be independent of tissue concentration within the limits of ordinary variability.

The possibility was considered that adenyl deaminase activity might release ammonia, which would interfere with the manometric observations. No increase in ammonia content of brain suspensions was found, however, by the nesslerization method from Umbreit *et al.* (25) after incubation of a relatively strong suspension (10 mgm./ml.) for an hour with 3 mM. ATP and 5 mM. magnesium.

Alberty *et al.* (1) have studied the dissociation constants of the adenylic acid derivatives and estimated the number of acid equivalents which should be released by the hydrolysis of ATP and ADP at various pH values. Results illustrated in Fig. 2 give somewhat lower ratios of carbon dioxide evolution to phosphate liberated than would be expected from their calculations. The

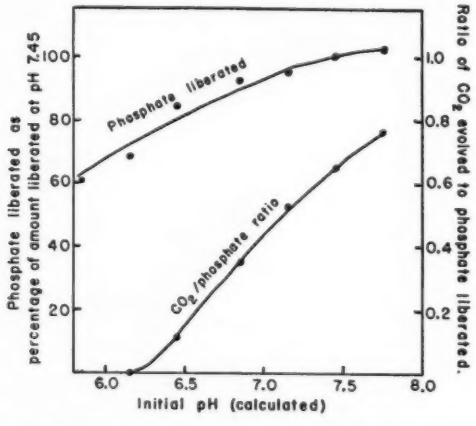

FIG. 2. Effect of pH on phosphate liberation and CO_2 evolution. Mixtures contained 3.3 mgm. tissue/ml., 3 mM. ATP, 5 mM. Mg. The required initial pH of the media was obtained by using various combinations of 12.5, 25, or 50 mM. bicarbonate with 5, 10, or 100% carbon dioxide in the gas phase. The initial pH was calculated from the constants summarized by Birmingham and Elliott (3). The pH fell slightly during the 60 min. experimental period due to the hydrolysis of ATP and other effects of the tissue.

The points shown are averages from several experiments in which there was some variability though the general result did not vary.

difference in ionization of the complexes of magnesium with ATP and inorganic phosphate, mentioned above, and some decrease in pH and increase in retention during the reaction and other factors would lower the carbon dioxide output. Under the standard conditions with magnesium as co-catalyst, the ratio of carbon dioxide equivalents released to the increase in inorganic phosphate averaged 0.61 ($\pm$ 0.05 standard deviation). The same average ratio was found after 15 min. incubation as after 60 min. The ratio approaches unity with increasing pH and no carbon dioxide evolution occurs at or below pH 6.15. From the values for chemically determined inorganic phosphate, it is apparent that the ATPase activity is considerable at pH 5.85 and increases with pH towards a maximum at about pH 8. The CO_2/P ratios obtained with calcium as co-catalyst are discussed below.

In the absence of added magnesium or calcium, appreciable activity is observed, presumably owing to small amounts of calcium in the tissue. Both magnesium and calcium ions stimulate ATP hydrolysis. In Fig. 3 the effects of magnesium and of ATP concentrations on the initial rates are illustrated. In the presence of a relatively low concentration of ATP (1.5 mM.), nearly maximum effect of magnesium occurs with 1 mM. Higher concentrations of ATP decrease the effectiveness of low magnesium concentrations and the maximum rate is reached only at higher magnesium concentrations. The maximum rate in either case is approximately the same. In the standard procedure, 3 mM. ATP and 5 mM. magnesium are used though perhaps 1.5 mM. ATP would be equally satisfactory and more economical.

With calcium as co-catalyst, a similar mutual relation between the concentrations of ATP and calcium affects the activity. At high calcium concentrations, however, a nonenzymatic artefact affects manometric readings.

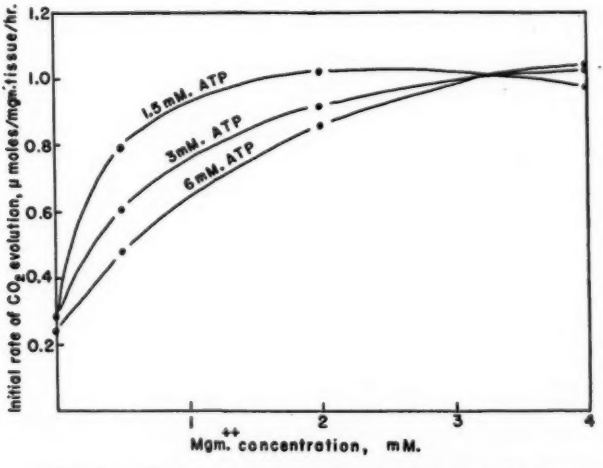

FIG. 3. Effects of ATP and magnesium concentrations.

The initial rate of carbon dioxide evolution is nearly constant with 2 to 4 mM. calcium but as the calcium concentration is further increased the rate increases again to another maximum, approximately double that with 4 mM. calcium, while the rate of phosphate liberation does not increase and even falls slightly at concentrations above 10 mM. Experiments in the absence of tissue showed that the extra carbon dioxide evolution was not connected with precipitation of ATP by calcium though this occurs, but could be accounted for by formation, from the phosphate enzymatically released, of calcium phosphate with release of hydrogen ions. As a result the carbon dioxide/inorganic phosphate ratio increases with increasing calcium concentration. Even with relatively low calcium concentrations the ratio is slightly higher than with magnesium. In the standard manometric procedure, with calcium as catalyst, 2 mM. calcium is used with 3 mM. ATP. No cloudiness appears and the carbon dioxide/inorganic phosphate ratio is about 0.72 ($\pm$ 0.04 standard deviation).

With adenosine diphosphate (ADP) as substrate and either magnesium or calcium, the rate of carbon dioxide evolution or of production of inorganic phosphate was approximately half of that with ATP (see Fig. 1). With adenylic acid (AMP) as substrate and magnesium as co-catalyst, inorganic phosphate is released (about 0.28 μmole/mgm. solidus hr.) with concomitant slow carbon dioxide *absorption* (at pH 7.4). The presence of AMP, 1.5 mM., with ATP decreased the initial carbon dioxide evolution obtained with ATP alone by about 30% and also decreased the total initial release of inorganic phosphate by 20%. Evidently AMP inhibits the ATPase appreciably. However under standard conditions the concentration of AMP which would be produced from ATP would be too low to interfere appreciably with the initial ATPase activity. The presence of added inorganic phosphate, up to 10 mM., had no appreciable influence on the rate of hydrolysis of ATP.

Results with inorganic pyrophosphate (PP) are given in Table I. In the presence of 5 mM. magnesium, inorganic phosphate is produced from PP at a lower rate than from ATP. Since hydrolysis of a single bond in PP releases

TABLE I

PHOSPHATE LIBERATION FROM ATP AND PP BY RAT WHOLE HEMISPHERE SUSPENSIONS

μmoles inorganic phosphate liberated per 100 mgm. tissue in 60 min.
(in 15 min. in parenthesis)

	ATP	PP*	ATP + PP
Mg, 5 mM.	(31) 87	(17) 47	(39) 126
	(24) 59	(18) 44	(39) 109
Ca, 2 mM.	(20) 59	(1) 2	(14) 46
	(21) 58	(0) 3	(20) 59

** Inorganic phosphate liberated from PP in other experiments with magnesium ranged from 0.40 to 0.56 μmoles/mgm./hr.*

two molecules of phosphate, the actual action rate is much lower than with ATP. (Little or no carbon dioxide evolution accompanies PP hydrolysis at the pH of these experiments.) When both ATP and PP are present, with magnesium as co-catalyst, the total inorganic phosphate liberated is approximately the sum of the amounts obtained with either alone. No stimulation of PP hydrolysis is produced by calcium and there is no additive effect of pyrophosphate and ATP with calcium as co-catalyst.

The release of inorganic phosphate from ATP in the presence of the optimum magnesium concentration is inhibited by the addition of calcium. Half-maximal inhibition is obtained with about 1 mM. calcium and, with high calcium concentrations, the rate obtained is equal to that given by the calcium alone. The hydrolysis of inorganic pyrophosphate, catalyzed by magnesium, is also strongly inhibited by calcium. Fifty per cent inhibition occurs with about 1 mM. calcium and almost complete inhibition with 10 mM. These results are illustrated in Fig. 4.

The results shown in Table I and Fig. 4, indicate that at least three enzymes are concerned. These are (*a*) an enzyme which hydrolyzes ATP, requires magnesium as co-catalyst, and is inhibited by calcium, (*b*) an enzyme which hydrolyzes ATP and requires calcium as co-catalyst, (*c*) an enzyme which hydrolyzes PP, requires magnesium as co-catalyst, and is inhibited by calcium. It is probable that adenylate kinase is concerned in the splitting of the second high energy phosphate group from ATP in these suspensions. Gore (11) and Gordon (10) have presented considerable evidence which indicates that PPase is not identical with ATPase. The calcium activated ATPase of myosin is well-known. Kielley and Meyerof (15) described and purified an ATPase from muscle which is activated by magnesium and inhibited by calcium. Inhibition of brain PPase by calcium has been observed by Gordon (9, 10).

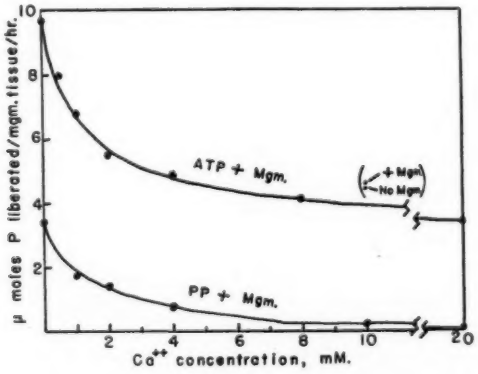

FIG. 4. Effects of calcium on the rate of hydrolysis of ATP and of PP in the presence of magnesium, 5 mM. Tissue 3.3 mgm./ml.; experimental period one hour. The points in parenthesis, obtained in a separate experiment, show that, at 10 mM. calcium, the presence or absence of magnesium has no effect.

The suspensions contain very active creatine transphosphorylase activity. Creatine phosphate alone is not attacked but when both creatine phosphate and ATP are present, with magnesium or calcium, chemical and manometric determinations show that the ATP concentration is maintained while inorganic phosphate is released indirectly from the creatine phosphate with increase in base. When an amount of phosphate equivalent to the added creatine phosphate has been released, the ATP concentration begins to fall and acid groups are produced.

Slices of cortex hydrolyze ATP in the suspending medium but not as rapidly as does disintegrated tissue. The rate, measured chemically or manometrically, is strongly dependent upon the ATP concentration in the medium. In the presence of 5 mM. magnesium in isotonic saline containing 1.5, 3.0, and 6.0 mM. ATP, 0.21, 0.29, and 0.44 micromoles of phosphate were released in 60 min. per mgm. of tissue. The rate is presumably limited mainly by the concentration within the slice which would depend upon the thickness of the slice, and the rates of diffusion into, and hydrolysis within, the slice. Suspensions prepared in isotonic medium (120 mM. sodium chloride – 25 mM. sodium bicarbonate) showed slightly lower activity than suspensions in hypotonic medium (bicarbonate alone). Homogenizing for an extra five minutes or repeated freezing and thawing of hypotonic suspension produced no further increase in activity. Meyerhof and Wilson (19) found that most of the "apyrase" activity of brain was present in insoluble particles. In accordance with this, we find that the residue produced on centrifugation of isotonic or hypotonic suspensions at about 20,000 × g. contains about 90% of the ATPase activity, as measured with magnesium or calcium as co-catalyst, and very little is found in the supernatant fluid.

With a purified enzyme preparation from brain, Binkley and Olson (2) found that maximum hydrolysis of PP was obtained only after incubation of the enzyme with glutathione or cysteine. With our fresh unfractionated suspensions the rate of hydrolysis of ATP (magnesium or calcium) or of PP (with magnesium) was not increased by addition of neutralized glutathione, 2 mgm. per ml., to the reaction mixture or by preincubation of the tissue suspension for 40 min. with neutralized glutathione. The ATPase activity was remarkably stable since incubation of the tissue anaerobically for 45 min. without substrate had no effect on the subsequent activity and preincubation under 95% oxygen caused only slight inactivation. The presence of oxygen during the hydrolysis had no effect. Pyrophosphatase activity was decreased about 50% by anaerobic preincubation.

Kielley and Kielley (14) and Potter *et al.* (22) have found that the ATPase activity of liver mitochondria or whole liver suspensions prepared in cold isotonic sucrose solution is activated by warming. Our whole-brain suspensions when prepared and kept below 2° C. showed the same activity as samples which had been warmed to 38° for 15 min. (The activity in these tests was measured at 25° instead of 38° to avoid the prewarming involved in equilibration at 38°.) The same result was obtained when the suspension was prepared in isotonic sucrose as in the usual dilute bicarbonate.

Swanson (23) has reported that the complexing agent ethylenediamine tetraacetate (EDTA, Versene) at low concentration caused acceleration of liver PPase while inhibition occurred with a concentration equivalent to that of the magnesium. Similar observations were made by Gross (13) with the calcium activated ATPase of heart myosin. Experiments recorded in Table II show that 3 mM. EDTA almost completely inhibits the ATPase activity of brain suspension in the absence of added ion or in presence of 2 mM. calcium. In the presence of 5 mM. magnesium, 7.5 mM. EDTA causes only partial inhibition and 1 mM. EDTA accelerates. When both magnesium, 5 mM., and calcium, 2 mM. are added the further addition of EDTA, 3 mM., causes an acceleration. As mentioned earlier, calcium inhibits the Mg-ATPase so that, in the presence of both ions, most of the activity is due to the Ca-enzyme the activity of which is less than that of the Mg-enzyme. When EDTA is also added, it evidently prevents calcium from activating its enzyme but it also prevents the calcium from inhibiting the Mg-enzyme; the activity of the Mg-activated enzyme therefore reappears.

TABLE II

EFFECTS OF MAGNESIUM, CALCIUM, AND ETHYLENEDIAMINE TETRAACETATE (EDTA)

μmoles per 100 mgm. tissue in one hour

	No ion		2 mM. calcium		5 mM. magnesium			2 mM. calcium + 5 mM. magnesium	
	—	EDTA 2 mM.	—	EDTA 3 mM.	—	EDTA 1 mM.	EDTA 7.5 mM.	—	EDTA 3 mM.
P liberated			35	1	53	67		43	61
CO_2 evolved*	9	0	21	5	31	34	13	27	32

* *Mean of three agreeing experiments.*

Fluoride, 20 mM., inhibited the magnesium and calcium activated systems by about 85 and 60% respectively.† The calcium activated system was inhibited by oxalate as strongly as by fluoride and less actively by citrate. A number of other substances of biochemical or pharmacological interest were tested on the magnesium activated system. *d*-Tubocurarine produced 30 to 40% inhibition at 1 mM. concentration. No marked effect was observed with 3 mM. 2,4,dinitrophenol, 1 mM. atropine, 20 mM. glutamine, or 1 mM. pentobarbital. Tetraethyl pyrophosphate, 7 mM., caused 40% inhibition and this was not accounted for by the ethylene glycol solvent.

† *With brain suspended in the isotonic bicarbonate buffered Ringer-type medium usually used in this laboratory, which contains 1.3 mM. calcium and 1.2 mM. magnesium, the initial ATPase activity was about 0.65 micromoles of inorganic phosphate per mgm. per hour and 20 mM. fluoride inhibited by only about 25%. The activity without fluoride is largely due to the calcium only, since the magnesium enzyme would be inhibited by the calcium. The activity in the presence of fluoride might be partly due to the magnesium enzyme since the inhibition of this enzyme by calcium would probably be prevented by fluoride as is the case with EDTA.*

In a study of the ATPase of guinea-pig brain suspensions, mostly in glycylglycine buffer solution, Gore (11) reported that the ATPase was not stimulated by calcium, that the Mg-activated enzyme was stimulated by potassium (about 8 mM. optimum) and that the activity was considerably greater in glycylglycine than in bicarbonate buffered medium. We have not been able to confirm these observations. Like DuBois and Potter (5) and Gordon (10) we find a definite effect of calcium, in either medium, though not as great as that of magnesium. The presence of potassium, 10 mM., had no effect on either the Ca- or Mg-activated enzyme in either buffer. (Substituting the potassium salts for the sodium salts of ATP, bicarbonate and chloride had no effect on the initial rate though the later course of the reaction was somewhat slower with potassium salts.) Equal activity was found with the two buffers at pH 7.4 with either calcium or magnesium.

The Standard Procedure and Results

Sets of determinations were run with two different quantities of tissue, 5 and 10 mgm. for mouse and 10 and 20 mgm. for larger animals, to ensure obtaining a suitable rate and to check constancy of activity per unit weight of tissue. All solutions were made up in 25 mM. bicarbonate solution which had been gassed with carbon dioxide. Three pairs of Warburg vessels contained, respectively, no added ion, 2 mM. $CaCl_2$, or 5 mM. $MgCl_2$. All contained 3 mM. sodium ATP and bicarbonate solution to make a final total volume of 3 ml. One of each pair received 0.3 ml. of the tissue suspension in the side arm, the other received 0.6 ml. After gassing with nitrogen —5% carbon dioxide at room temperature and equilibration at 38°, the tissue was tipped in at zero time and readings were taken at frequent intervals; the carbon dioxide evolution in 15 min. was taken from the time–evolution curve.

Results are summarized in Table III. There was considerable variability in the activity of tissue from different individual animals and periods when all

TABLE III

AVERAGE INITIAL ATPASE ACTIVITIES

μmoles P liberated/100 mgm. tissue/hr.—averages and ranges

	Number of determs.	No ion	Magnesium	Calcium
Mouse whole hemisphere	2	37, 37	158, 161	72, 79
Rat whole hemisphere	35	27 ± 9*	142 ± 24*	70 ± 10*
Mouse cortex	6	31 (27–37)	175 (149–205)	78 (69–87)
Rat cortex	5	37 (30–42)	153 (123–173)	83 (69–94)
Cat cortex	5	25 (15–35)	134 (103–164)	54 (47–62)
Beef cortex	6	17 (15–22)	87 (79– 97)	27 (22–30)
Human cortex	14†	18 (12–32)	62 (47– 82)	34 (22–50)

Results calculated from the carbon dioxide evolved in 15 min. and corrected for the mean CO_2/P ratio (0.61 with magnesium, 0.72 with calcium).

* *Standard deviation.*

† *See footnote to text.*

values for rats were relatively low. These fluctuations were not due to changes in technique or reagents and are unexplained. Large variations in PPase activity of brain suspensions have been reported by Gordon (10). Results with tissue from a number of rats anesthetized with pentobarbital were not different from those obtained at the same time with tissue from untreated animals. The activity of suspensions of rat or mouse cortical gray matter was about the same as, or slightly higher than, that of whole hemisphere suspensions.

In Fig. 5, *a* and *b*, the average activities found with cortical gray matter from various animals have been plotted against the approximate body weight (*a*) or the brain weight (*b*) on double logarithmic scales. The figures for human brain were obtained with tissue excised during operations by Dr. Wilder Penfield. There is an obvious decline in average activity per unit weight with size of the species. The regression lines shown were calculated by

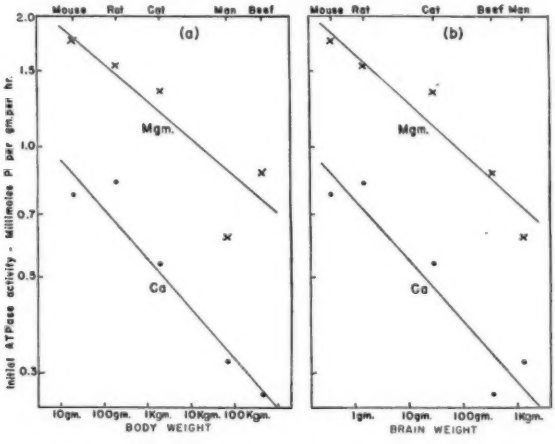

FIG. 5. Relation of ATPase activity to (*a*) body weight or (*b*) brain weight of different mammals with magnesium or calcium as co-catalyst. Logarithmic scales. The brain weights in grams are taken as mouse 0.4, rat 1.6, cat 31, beef 375, man 1320 (Count (4)).

the method of least squares from all the individual determinations‡. These lines represent the relation $A = KW^{-k}$ between initial activity, A, (in millimoles of P liberated per gm. of tissue per hour) and brain or body weight, W, (in grams) and $-k$ is the regression coefficient. For the Mg-ATPase the relations are $A = 1.64\ W^{-0.11}$ for brain weight and $A = 2.3\ W^{-0.09}$ for body weight. For the Ca-ATPase the relations are respectively $A = 0.76\ W^{-0.14}$ and $A = 1.23\ W^{-0.12}$.

‡ *For human brain only the values obtained with four normal samples were used in these calculations. The figures in Table III cover also a number of samples of human tissue which were abnormal but which showed the same average ATPase activities as the normal samples and also were found to show normal respiratory activity (Pappius and Elliott, 21).*

The correlations are highly significant in all cases but the Mg-activated system is more closely related to the brain weight while the Ca-activated system is more closely related to the body weight. This difference is apparent in Fig. 5 particularly when the results for beef and human brain are compared.

Similar double logarithmic relations have been shown between the rate of oxygen consumption of slices of cerebral cortex and body weight (7, 8) and between various activities of the acetylcholine system and brain weight (24).

The regression coefficient, k, for oxygen consumption was about -0.1, which is about the same as for ATPase activity, while the coefficient for all the activities of the acetylcholine system was about -0.3, indicating a much sharper decline with increasing size.

The oxygen uptake rate for slices of gray matter, like the ATP splitting activity, varies considerably between individuals of a species. It also varies with the constitution of the surrounding medium. Average rates reported for rat cortex slices vary between 200 micro atoms of oxygen per gm. per hour, in a medium resembling spinal fluid (8), and about 400§ in Krebs' (16) supplemented but calcium-free medium. Work by various authors has indicated that about three molecules, or somewhat more, of inorganic phosphate can be esterified into the form of high energy phosphate per atom of oxygen consumed. Thus the possible rate of high energy phosphate production by rat cortex would be about 600 to 1200 micromoles per gm. per hour. The average initial rate of phosphate release from ATP by rat cortical tissue under optimal conditions with magnesium as co-catalyst is about 1500 micromoles per gm. per hour. Thus the ATP splitting activity about equals or somewhat exceeds, the upper level of the potentiality to produce high energy phosphate. Since the regression lines for rates of oxygen uptake and ATP splitting are approximately parallel, that is k is about the same for both, similar calculations give similar results for all species.

By comparing rates obtained with slices and suspensions with rates found by others for the whole brain *in vivo*, it has been concluded (6, 7) that the upper level of *in vitro* results might correspond to the rate of oxygen consumption by brain tissue *in situ* in the conscious, normal subject. If three molecules of high energy phosphate are produced per atom of oxygen consumed and if conditions for ATP hydrolysis are optimal *in vivo*, the rates of formation of high energy phosphate and its destruction via ATP hydrolysis would just about balance.

It seems unlikely that the release of inorganic phosphate in our suspensions is accomplished to any appreciable extent indirectly by transphosphorylation with subsequent breakdown of the esters produced. No glucose or other substance known to be readily phosphorylated is present. The release is mostly accomplished by true ATPase, probably in conjunction with adenylate kinase. ATPase activity is connected with the contractile process in muscle but in other tissues its role is unknown. The fact that the Mg-activated

§ We (21) do not find such high rates as does Krebs with his supplemented medium probably largely because we use the initial weight, rather than the dry weight of tissue remaining after incubation, as basis of calculation.

ATPase activity in the various species seems to be correlated with respiratory activity, a continuous function, suggests that this ATPase may be mainly connected with the maintenance of a continuous cell condition such as the normal intracellular cationic concentrations. The Ca-activated ATPase might also be concerned in continous function but it seems likely that, as in muscle, it is also concerned in the mechanism of the specific function of the tissue. From studies on the activities of cytochrome oxidase and ATPase in cyto-architectonic layers of rat cortex, Hess and Pope (13a) have concluded that separate Ca- and Mg-activated ATPase are present with different cytological locations. The Mg-activated enzyme has a similar distribution to the respiratory enzyme cytochrome oxidase and the Ca-activated enzyme has a distribution resembling that of acetylcholinesterase.

References

1. ALBERTY, R. A., SMITH, R. M., and BOCK, R. M. J. Biol. Chem. 193 : 425. 1951.
2. BINKLEY, F. and OLSON, C. K. J. Biol. Chem. 186 : 725. 1950.
3. BIRMINGHAM, M. K. and ELLIOTT, K. A. C. Can. J. Med. Sci. 30 : 403. 1952.
4. COUNT, E. W. Ann. N.Y. Acad. Sci. 46 : 993. 1947.
5. DUBOIS, K. P. and POTTER, V. R. J. Biol. Chem. 150 : 185. 1943.
6. ELLIOTT, K. A. C. The biology of mental health and disease. Paul B. Hoeber, Inc., New York. 1952.
7. ELLIOTT, K. A. C. *In* Neurochemistry *Edited by* ELLIOTT, K. A. C., PAGE, I. H., and QUASTE, J. H. Charles C. Thomas. Springfield. In press. 1954.
8. ELLIOTT, K. A. C. and HENDERSON, N. J. Neurophysiol. 11 : 473. 1948.
9. GORDON, J. J. Biochem. J. (London), 46 : 96. 1950.
10. GORDON, J. J. Biochem. J. (London), 55 : 812. 1953.
11. GORE, M. R. B. Biochem. J. (London), 50 : 18. 1952.
12. GREEN, I. and MOMMAERTS, W. F. H. M. J. Biol. Chem. 202 : 541. 1953.
13. GROSS, M. Science, 118 : 218. 1953.
13a. HESS, H. E. and POPE, A. Federation Proc. 13 : 228. 1954.
14. KIELLEY, W. W. and KIELLEY, R. K. J. Biol. Chem. 191 : 485. 1951.
15. KIELLEY, W. W. and MEYERHOF, O. J. Biol. Chem. 176 : 591. 1948.
16. KREBS, H. A. Biochim. et Biophys. Acta, 4 : 249. 1950.
17. MCELROY, W. D. and GLASS, B. Phosphorus metabolism. The Johns Hopkins Press. Baltimore. 1951.
18. MCLENNAN, H. and ELLIOTT, K. A. C. Arch. Biochem. and Biophys. 36 : 89. 1952.
19. MEYERHOF, O. and WILSON, J. R. Arch. Biochem. 14 : 71. 1947.
20. NACHMANSOHN, D. and JOHN, H. M. J. Biol. Chem. 158 : 157. 1945.
21. PAPPIUS, H. and ELLIOTT, K. A. C. Can. J. Biochem. Physiol. 32 : 484. 1954.
22. POTTER, V. R., SIEKEVITZ, P., and SIMONSON, H. C. J. Biol. Chem. 205 : 893. 1953.
23. SWANSON, M. A. J. Biol. Chem. 194 : 685. 1952.
24. TOWER, D. B. and ELLIOTT, K. A. C. Am. J. Physiol. 168 : 747. 1952.
25. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and tissue metabolism. Burgess Publishing Company, Minneapolis. 1949.

ADENOSINE TRIPHOSPHATASE, ELECTROLYTES, AND OXYGEN UPTAKE RATES OF HUMAN NORMAL AND EPILEPTOGENIC CEREBRAL CORTEX[1]

By Hanna Pappius and K. A. C. Elliott

Abstract

The magnesium-activated and the calcium-activated adenosine triphosphatase activities, the potassium and sodium content, and the oxygen uptake rate in a medium containing glucose, pyruvate, glutamate, and fumarate have been determined in human cerebral cortex excised from epileptic patients. No differences between focal epileptogenic and normal tissue were detected.

Introduction

This study is one of a series in which advantage is taken of the opportunity to obtain human brain tissue freshly excised from unanesthetized epileptic patients undergoing neurosurgical operations in this Institute. The biochemical concomitants of any activity which varies from moment to moment, such as epileptoid activity, cannot be studied on human material by methods now available. Chronic biochemical abnormalities in epileptogenic tissue which may underlie the tendency to initiate seizures can, however, be sought. Samples of definitely normal tissue from unanesthetized patients for study as normal controls become available only rarely. This difficulty has been fairly satisfactorily overcome by an indirect method. Similar determinations are done on normal tissue of the same type from various mammalian species and the results are plotted against body weight or brain weight on an appropriate scale. From the curves obtained a probable average value for normal human brain can be obtained by extrapolation or interpolation; the variability found in other species indicates the probable variability of normal values for human tissue. Figures for various activities obtained with actual samples of normal human brain have accorded well with the indirectly estimated values in the present and previous studies (2, 12, 9).

In previous studies it was shown that the respiratory and glycolytic abilities of epileptogenic tissue were not necessarily abnormal (6). No significant difference between epileptogenic and nonepileptogenic tissue was found in total acetylcholine content or in the rate of synthesis of acetylcholine by slices on incubation in suitable media. But the epileptogenic tissue showed a tendency to have an elevated cholinesterase activity and slices showed a failure to produce bound acetylcholine on incubation (13).

The present report records the results of a search for abnormality in three further aspects of brain biochemistry namely (*a*) two kinds of adenosine triphosphatase activity, (*b*) potassium and sodium content, and (*c*) oxygen uptake rate in the supplemented medium of Krebs (8) which gives rates which are higher than those obtained in the usual media.

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Contribution from the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montreal, Que.

Methods

Samples of cerebral cortex, excised *en bloc*, were obtained from epileptic patients under local nupercaine anesthesia during operations by Dr. Wilder Penfield for focal cortical seizures. The epileptogenic activity of each sample was rated according to its electrical activity and response to stimulation *in situ* and its gross appearance. Further details and notes on the types of samples obtained are given in preceding papers (6, 13). Most of the human samples were from the temporal lobe. The experimental animals were decapitated instantaneously without anesthesia and the brains were removed at once.

The excised tissue was placed in a dry dish which was put in a humid chamber. Major membranes and blood were removed with forceps and filter paper; the pia was removed from human brain samples. Portions of gray matter were dissected off, 100 mgm. or more for the ATPase and 150 mgm. or more for the electrolyte determinations, and slices of gray matter were prepared by means of the Stadie–Riggs (11) microtome for the oxygen uptake determinations. The ATPase activity and the respiration rate were measured without delay.

ATPase Activity

This was determined at 38° by the manometric method of Pappius and Elliott (9), without added ion, with 5 mM. magnesium and with 2 mM. calcium, on tissue homogenized in bicarbonate buffer solution. Two concentrations of tissue, 10 and 20 mgm. in 3 ml., were used and the results, which showed no consistent difference, were averaged.

Potassium and Sodium

For these determinations the tissue was weighed on an aluminum pan and stored on the pan in a deep freeze cabinet. When convenient, the frozen tissue was introduced into a small stainless steel homogenizer and homogenized with water. The suspension was quantiatively made up to 50 ml. with water and 2 ml. of 1.25% lithium (as nitrate). A small drop of octanol was used to eliminate foam. The suspension was centrifuged and the supernatant fluid was filtered through Whatman No. 40 filter paper. Potassium and sodium were determined on the filtrate by the internal standard method using the Perkin–Elmer flame photometer and standard solutions containing the same concentration of the lithium salt as in the unknown and similar concentrations of potassium and sodium. A blank determination was always carried out with water from the same bottle, "homogenized" in the same apparatus and carried through the full procedure. Corrections for the blank value, usually zero for potassium but appreciable for sodium, were applied to the unknowns.

The reliability of the above simple method was tested as follows. Four samples of cortex were prepared from a cat brain. Samples from the frontal and occipital regions were analyzed by the simple procedure. Two samples

from intermediate areas were dissolved in concentrated nitric acid. The acid was evaporated off on the steam bath and the residue was warmed and triturated with 2 ml. of ether and water and finally made up to volume with water and lithium nitrate solution, filtered, and analyzed. Simple and nitric acid blanks were run similarly. The results were as follows, in milli-equivalents per kilogram.

	Potassium	Sodium
Simple procedure	92.7, 92.5	67.1, 67.5
Nitric acid digestion	92.8, 92.1	68.8, 71.5

Respiration Rate

Conventional Warburg apparatus was used. The medium was Krebs medium II (8) which is an isotonic, phosphate-buffered, calcium-free, saline mixture containing glucose, 9 mM., and pyruvate, fumarate, and glutamate, each 5 mM. (This solution was stored in the frozen state.) The gas phase was oxygen and the center wells of the vessels contained alkali-soaked filter paper. The slicing instrument cut slices about 0.5 mm. thick. A surface slice and a second slice from each sample were set up separately. The results with the two slices sometimes differed by as much as 32% but there was no consistent difference in activity between the surface and the deeper slice (see also (2)) and the figures given show the means of the activities of the two slices.

Results and Discussion

Adenosine Triphosphatase

Tower and Elliott (14) reported that an abnormality in production of bound acetylcholine, which is found in slices of epileptogenic tissue and in normal slices subjected to incomplete oxygenation, could be corrected by glutamine but not by glutamate. It is known that glutamine can be produced from glutamic acid and ammonia by an enzyme system which requires adenosine triphosphate (ATP) (7, 10). It seemed possible that a lack of the ATP necessary for the production of glutamine from endogenous glutamate might cause the abnormality in acetylcholine metabolism and Tower and Elliott reported preliminary experiments which showed that added ATP in the surrounding medium would correct the abnormality which is found in incompletely oxygenated slices. The possibility that the activity of the enzymes (ATPases) which hydrolyze ATP might be elevated in epileptogenic tissue was therefore considered. Determinations of ATPase activity seemed worth while also because ATP hydrolysis is intimately concerned in the mechanism of muscular contraction and it is probably concerned in the mechanisms of nervous activity. Pappius and Elliott (9) have shown that the ATPase activity in brain is high enough to liberate all the high energy phosphate which could be generated by the respiring tissue.

The activities of the magnesium-activated and the calcium-activated ATPase in tissue from epileptic patients have therefore been determined.

TABLE I

ADENOSINE TRIPHOSPHATASE

(Ml. of CO_2 liberated per gram of fresh tissue per hour during the first 15 min.)*

	Number of samples	No ion	Magnesium	Calcium
		Average (range)	Average (range)	Average (range)
Probable focus	6	2.8 (2.4–4.4)	8.5 (7.6– 9.2)	5.4 (4.4–6.0)
Possible focus	4	2.8 (2.4–3.2)	8.5 (6.4–11.6)	5.9 (5.2–8.0)
Normal	4	2.4 (2.0–3.2)	8.3 (6.6–11.2)	5.1 (3.4–6.4)
Predicted normal	—	2.9 approx.	9.9 approx.	5.1 approx

The above figures include one case in which a sample of normal tissue and a sample of probable focal tissue were obtained from the same patient and showed very nearly the same activities.

** The rate of inorganic phosphate liberation in micromoles/100 mgm./hr. is obtained approximately by dividing by 22.4×10^{-3} and by 0.61 for the magnesium results or 0.72 for the calcium results (9).*

The results shown in Table I indicate that there is no consistent difference between the activities found in epileptogenic tissue and those found in samples of probably normal tissue. Averages of these results and of results obtained with tissue from various normal experimental animals, are plotted in Fig. 5 of Pappius and Elliott (9). This figure shows that there is a relation between the mean magnesium-activated ATPase activity and the brain weight of the species and there is a relation between the calcium-activated ATPase and the body weight of the species. From these relations an approximate mean value for the activities of normal human tissue can be predicted. The activities found with epileptogenic tissue samples are not significantly different from predicted normal values.

Potassium and Sodium Content

It is well known that potassium is lost and sodium is gained by nerve or muscle during activity. In abnormal nervous tissue in which there is continually a greater degree of activity than normal it seemed possible that the electrolyte content of the whole tissue might become abnormal. Conversely, some abnormality in the metabolism of the tissue resulting from impaired local circulation or due to defect in the cellular mechanisms might cause an abnormal distribution of electrolytes which could be the cause of the hyperactivity and which might be detectable as a change in the electrolyte content of the whole tissue.

In view of these possibilities, determinations have been made of the potassium and sodium content of cortical gray matter from epileptic patients

TABLE II

POTASSIUM AND SODIUM CONTENT

(Milliequivalents per kgm. of fresh tissue)

	Number of samples	Potassium	Sodium		Number of samples	Potassium	Sodium
	Human				Cat		
Probable focus	6	98 (90–103)	60 (55–68)		6	99 (93–105)	63 (56–68)
Possible focus	4	98 (89–105)	62 (53–73)		Rat		
Normal	3	97 (91–101)	62 (60–66)		5	104 (101–106)	56 (49–63)
					Rat (convulsed)*		
					8	104 (101–105)	48 (44–55)

Averages (ranges in parentheses).

** Convulsions induced by three second shocks of 40 volt 60 cycle applied by electrodes to shaved areas near the ears. Animal killed during or immediately after the convulsion.*

and from normal experimental animals. The results, presented in Table II, show that the concentration of potassium, and probably that of sodium, does not vary significantly from species to species and that all the human samples contained apparently normal concentrations of potassium and sodium. Cortex from a cat suffering methionine sulphoximine-induced convulsions also gave normal values. The variability in the results is probably partly due to technical factors. A piece of brain immersed in a saline medium under conditions unfavorable for proper metabolism rapidly loses potassium and gains sodium. Excision of the tissue involves a variable period of interference with circulation and the brain samples are usually covered with blood removal of which takes time.

It should be emphasized that these determinations could not disclose abnormalities of distribution between intra-, and extracellular spaces unless these resulted in an exchange of the electrolytes between the tissue as a whole and the plasma or cerebrospinal fluid. This could probably only occur if the condition persisted continuously for a considerable period. Electrically induced seizures did not produce any change in the electrolyte content which was clearly detectable by the present method (Table II) though Colfer and Essex (1) have shown, by micro-incineration, that changes in the distribution of electrolytes between neurones and their immediate environment occur in brain during convulsions.

Respiration in Krebs Supplemented Medium

In a previous report (2) it was shown that epileptogenic tissue could respire and glycolyze in ordinary saline media at normal rates though an occasional very gliosed specimen showed very low metabolic activity. Since most of the focal epileptogenic brain samples were somewhat tough, it seemed advisable to check their respiratory activity since possible changes in the other

factors determined might be due simply to replacement of brain by scar tissue. The respiratory activity of slices was measured in the supplemented phosphate-buffered medium of Krebs (8) in order to determine whether this medium might show a difference between epileptogenic and normal tissue which was not apparent when the conventional media were used (2). The results, which are summarized in Table III, show no significant difference between normal and epileptogenic tissue and the rates obtained for human tissue were about what would be predicted from results with tissue from cat and beef.

TABLE III

OXYGEN CONSUMPTION OF SLICES

In Kreb's supplemented phosphate-Ringer solution

(Milliliters per gram of fresh tissue per hour)

	Number of samples	O_2 uptake	Number of animals	O_2 uptake
	Human		Rat	
Probable focus	5	2.5 (2.3–2.9)	3	3.3 (3.0–3.8)
Possible focus	3	2.2 (2.1–2.4)		
			Cat	
Normal	4	2.4 (1.8–3.0)	3	3.7 (3.0–4.3)
Predicted normal*		2.2 approx.		
			Beef	
			2	1.4, 1.7

* *Calculated from results obtained with cat and beef by applying the equation: rate = constant* $\times W^{-0.1}$ *where W is the body weight (2, 4).*

Taking the fresh weight/dry weight ratio as 5, the present results for rat, cat, and beef are lower than those of Krebs for the same medium. This is probably largely due to the fact that the present results were based on the initial weight of tissue instead of on the dry weight determined on the tissue removed from the medium after the incubation (see (5)). The rates were not greatly or uniformly higher than figures obtained previously (2) by the same technique but with conventional medium containing no organic substance other than glucose. Earlier experience ((5) and unpublished) had shown that, in the presence of glucose, addition of pyruvate, fumarate, or glutamate, singly, did not greatly affect brain tissue slice respiration and it is difficult to understand why very high rates should be expected when they are added together.*

* *Krebs' results, like those of Elliott (2) showed wide variability but a fairly regular decrease in average activity with the size of the species (see (4, 3)), but in the particular medium used in this work the average for rat cortex was lower than for cat and some other larger animals; this anomaly seems apparent also in our results.*

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References

1. COLFER, H. F. and ESSEX, H. E. Am. J. Physiol. 150 : 27. 1947.
2. ELLIOTT, K. A. C. J. Neurophysiol. 11 : 473. 1948.
3. ELLIOTT, K. A. C. *In* The Biology of Mental Health and Disease. Paul B. Hoeber, Inc., New York. 1952.
4. ELLIOTT, K. A. C. *In* Neurochemistry. *Edited by* K. A. C. ELLIOTT, I. H. PAGE, and J. H. QUASTEL. Charles C. Thomas, Publisher, Springfield, Ill. 1954. In press.
5. ELLIOTT, K. A. C., GREIG, M. E., and BENOY, M. P. Biochem. J. (London), 31 : 1003. 1937.
6. ELLIOTT, K. A. C. and PENFIELD, W. P. J. Neurophysiol. 11 : 485. 1948.
7. ELLIOTT, W. H. and GALE, E. F. Nature, 161 : 129. 1948.
8. KREBS, H. A. Biochim. et Biophys. Acta, 4 : 219. 1950.
9. PAPPIUS, H. and ELLIOTT, K. A. C. Can. J. Biochem. Physiol. 32 : 471. 1954.
10. SPECK, J. F. J. Biol. Chem. 179 : 1405. 1949.
11. STADIE, W. C. and RIGGS, B. C. J. Biol. Chem. 154 : 687. 1944.
12. TOWER, D. B. and ELLIOTT, K. A. C. Am. J. Physiol. 168 : 747. 1952.
13. TOWER, D. B. and ELLIOTT, K. A. C. J. Appl. Physiol. 4 : 669. 1952.
14. TOWER, D. B. and ELLIOTT, K. A. C. J. Appl. Physiol. 5 : 375. 1953.

EFFECT OF VITAMIN E ON CARBOHYDRATE METABOLISM OF RAT DIAPHRAGM[1]

By Doris E. Gray[2] and H. A. De Luca[3]

Abstract

A study was made of the carbohydrate metabolism of diaphragm isolated from rats maintained on diets containing deficient, sufficient, or excessive quantities of vitamin E, respectively. No effect was observed on glycogen formation, glucose uptake, lactic acid accumulation, or oxygen consumption as a result of differences in vitamin E intake. The data suggested, however, that diaphragm from rats receiving a diet deficient in vitamin E may have a slightly decreased capacity to form pyruvic acid or else a slightly increased ability to utilize it. The addition of insulin to the incubation medium counteracted this effect of the vitamin lack.

In a previous communication (4) the present investigators, on the basis of data obtained in the intact animal (dog), postulated that the intake of vitamin E influences the metabolism of carbohydrate. It was suggested that this vitamin might act synergistically with insulin, thus bringing about an increase in the peripheral utilization of glucose. In order to test this hypothesis, *in vitro* experiments were designed using diaphragm isolated from rats that had been fed diets containing deficient, sufficient, or excessive quantities of vitamin E.

Experimental Procedures

All animals used were rats of the Sprague-Dawley strain. In each experiment, three groups of rats of 100–150 gm. in weight were fed ad libitum for periods of four to six weeks on a diet the caloric composition of which was 22% protein, 27% fat, and 51% carbohydrate. The fat was supplied in the form of lard which is low in vitamin E. The other vitamins and minerals were added as supplements to the diet. The three groups of animals were designated as follows:

E-deficient; no vitamin E was given,

E-sufficient; daily dose of 0.5 mgm. vitamin E per rat,

E-excess; daily dose of 100 mgm. vitamin E per rat.

The vitamin E (*dl*-α-tocopheryl acetate) was administered orally by a calibrated dropper.

At the end of the maintenance period, one rat was taken from each of the groups under investigation and fasted for 24 hr. The animals were then killed by stunning and decapitation. The blood was collected for plasma tocopherol determinations. The diaphragm was dissected out with a minimum of trauma, placed on an ice-cold Petri dish, and trimmed in order to obtain two hemi-diaphragms of approximately equal weight. The hemi-diaphragms were then weighed on a torsion balance. One hemi-diaphragm

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was plunged into hot 30% (w/v) potassium hydroxide for determination of initial glycogen. The other hemi-diaphragm was placed in a Warburg flask containing 2 ml. of a phosphate–saline medium (10) to which had been added 2 mgm. glucose per ml. buffer. When the effect of the addition of insulin to the medium was under investigation, a similar procedure was followed; in this case, however, 0.5 units insulin (crystalline zinc 'Eli Lily') per ml. of medium was added to the contents of the flask.

The flasks were connected to manometers, oxygenated for 10 min., and incubated at 37° C. After equilibration for 10 min., the oxygen consumption was measured at 10-min. intervals for 90 min. Following incubation, the diaphragm was removed and placed in hot 30% (w/v) potassium hydroxide for determination of glycogen. The medium was then analyzed for the glucose remaining. From these determinations the glycogen formed and the glucose taken up by the diaphragm were calculated.

A similar procedure was followed in the estimation of lactic and pyruvic acids. It was observed that the concentration of each of these compounds in the diaphragm after incubation was less than the initial concentration; the medium, however, contained significant amounts of both lactic and pyruvic acids. Obviously diffusion of these compounds from the tissue had taken place. It was not possible, in the case of each of these two metabolites, to calculate both the amount formed and the amount metabolized. Only the difference between these two parameters (i.e. the "accumulated" amount of metabolite) could be estimated. Thus for pyruvic acid, the following calculation was made.

> Accumulated pyruvic acid = pyruvic acid in medium after incubation + pyruvic acid in tissue after incubation − pyruvic acid in tissue before incubation.

Estimation of the accumulated lactic acid was made in the same manner.

Glycogen and glucose determinations were made using the 'Anthrone' reagent described by Morris (7). Lactic acid was estimated by the method of Barker and Summerson (1); pyruvic acid by the method of Friedmann and Haugen (2). Plasma tocopherol was determined by the method of Quaife *et al.* (8).

Results

Tables I, II, III, and IV show that differences in the intake of vitamin E had no effect on (1) the initial glycogen content of the diaphragm (Table I), (2) glycogen found following incubation with glucose as substrate with or without added insulin (Table I), (3) glucose uptake with or without added insulin (Table I), (4) initial concentration of lactic acid (Table II), (5) accumulated lactic acid resulting from incubation with glucose as substrate, with or without added insulin (Table II), (6) oxygen consumption by the tissue during incubation with or without added insulin (Table III), (7) initial concentration of pyruvic acid (Table IV), and (8) pyruvic acid accumulated in the presence of insulin (Table IV).

TABLE I

GLYCOGEN AND GLUCOSE IN RAT DIAPHRAGM

	Experimental conditions					
	Vitamin E deficient		Vitamin E sufficient		Vitamin E excess	
	N	Mean ± S.E.M.	N	Mean ± S.E.M.	N	Mean ± S.E.M.
Initial glycogen	8	6.6 ± 0.30	8	6.3 ± 0.78	8	7.2 ± 0.72
Final glycogen (G)	16	11.0 ± 1.03	16	9.9 ± 0.69	16	11.9 ± 1.25
Glycogen formed	—	5.3 ± 1.07	—	3.6 ± 1.04	—	4.7 ± 1.44
Final glycogen (GI)	8	15.1 ± 1.38	8	15.4 ± 2.62	8	16.6 ± 2.46
Insulin effect	—	3.2 ± 1.72	—	5.5 ± 2.71	—	4.7 ± 2.76
Glucose uptake (G)	16	25.1 ± 1.48	16	22.1 ± 1.03	16	22.9 ± 1.82
Glucose uptake (GI)	8	32.4 ± 1.73	8	33.9 ± 3.52	8	30.7 ± 3.82

N is the number of animals.
G and I indicate presence of glucose and insulin, respectively, in the medium.
Estimations are expressed as μM. glucose per gram wet weight of tissue.
Insulin effect is the final glycogen (GI) − final glycogen (G).

TABLE II

LACTIC ACID IN RAT DIAPHRAGM

	Experimental conditions					
	Vitamin E deficient		Vitamin E sufficient		Vitamin E excess	
	N	Mean ± S.E.M.	N	Mean ± S.E.M.	N	Mean ± S.E.M.
Initial lactic acid	16	19.9 ± 1.08	16	19.0 ± 1.31	16	19.2 ± 1.74
Final lactic acid (G)	16	3.6 ± 0.45	16	4.2 ± 0.79	15	3.5 ± 0.53
Lactic acid in medium (G)	16	22.4 ± 1.96	15	24.9 ± 1.41	15	23.7 ± 2.14
Accumulated lactic acid (G)	—	6.1 ± 2.28	—	10.1 ± 2.08	—	8.0 ± 2.81
Final lactic acid (GI)	20	3.0 ± 0.34	20	2.7 ± 0.32	20	2.9 ± 0.34
Lactic acid in medium (GI)	15	22.6 ± 1.89	15	23.2 ± 1.51	15	24.2 ± 1.00
Accumulated lactic acid (GI)	—	5.7 ± 2.20	—	6.9 ± 2.02	—	7.9 ± 2.03

N is the number of animals.
G and I indicate presence of glucose and insulin, respectively, in the medium.
Estimations are expressed as μM. lactic acid per gram wet weight of tissue.

TABLE III

OXYGEN CONSUMPTION OF RAT DIAPHRAGM

	Experimental conditions					
	Vitamin E deficient		Vitamin E sufficient		Vitamin E excess	
	N	Mean ± S.E.M.	N	Mean ± S.E.M.	N	Mean ± S.E.M.
Oxygen uptake (G)	32	55.9 ± 2.09	32	55.2 ± 1.45	31	52.9 ± 1.58
Oxygen uptake (GI)	23	53.5 ± 2.44	23	51.5 ± 2.00	23	53.5 ± 2.15
Plasma tocopherol (vitamin E)	43	0	51	0.66 ± 0.25	40	1.82 ± 0.18

N is the number of animals.
G and I indicate presence of glucose and insulin, respectively, in the medium.
Oxygen uptake is expressed as μM. oxygen per gram wet weight of tissue per hour.
Plasma tocopherol is expressed as mgm. per 100 ml. plasma.

TABLE IV

PYRUVIC ACID IN RAT DIAPHRAGM

	Experimental conditions					
	Vitamin E deficient		Vitamin E sufficient		Vitamin E excess	
	N	Mean ± S.E.M.	N	Mean ± S.E.M.	N	Mean ± S.E.M.
Initial pyruvic acid	16	0.22 ± 0.03	16	0.23 ± 0.02	16	0.19 ± 0.02
Final pyruvic acid (G)	16	0.14 ± 0.02	15	0.13 ± 0.02	15	0.14 ± 0.02
Pyruvic acid in medium (G)	16	1.21 ± 0.09	16	1.52 ± 0.09	15	1.35 ± 0.08
Accumulated pyruvic acid (G)	—	1.13 ± 0.10	—	1.42 ± 0.09	—	1.30 ± 0.08
		($P < 0.02$)				
Final pyruvic acid (GI)	20	0.17 ± 0.02	20	0.16 ± 0.02	20	0.16 ± 0.03
Pyruvic acid in medium (GI)	15 ($P < 0.05$)	1.48 ± 0.08	15	1.39 ± 0.08	15	1.49 ± 0.07
Accumulated pyruvic acid (GI)	—	1.43 ± 0.09	—	1.32 ± 0.08	—	1.46 ± 0.08

N is the number of animals.
G and I indicate presence of glucose and insulin, respectively, in the medium.
Estimations are expressed as μM. pyruvic acid per gram wet weight of tissue.

Table IV, however, indicates that the accumulated pyruvic acid resulting from incubation of the tissue with glucose as substrate and without the addition of insulin was decreased by a low intake of vitamin E. The differences in the accumulated pyruvate between the three groups of animals were, however, relatively small and could be attributed almost entirely to the pyruvic acid in the medium. There was no significant difference in either the initial or final pyruvic acid concentrations in diaphragm taken from the three groups. It is interesting to note that the decreased pyruvate accumulation of the diaphragm from the E-deficient group could be raised to the normal level by the addition of insulin to the incubation medium, but no significant effect was noted in this regard in the case of the animals fed adequate or excessive amounts of the vitamin.

Tables I to IV indicate that there was no significant difference in any of the parameters measured between the animals of the vitamin E sufficient and vitamin E excess groups.

Discussion

Dietary levels of vitamin E had no influence on the metabolism of the diaphragm relative to the initial glycogen content, glycogen formation, or glucose uptake during incubation with either glucose or glucose and insulin. It would appear from these findings that this vitamin does not play a role in the series of reactions leading to the formation of glycogen from glucose. (This conclusion is not in agreement with that reached by Zierler *et al.* (11) who reported that injections of vitamin E in the form of alpha-tocopheryl phosphate resulted in a decrease in glycogenesis, although there was no effect on the uptake of glucose by the diaphragm.) On the other hand, incubation of tissue from animals receiving a diet deficient in vitamin E resulted in a decreased accumulation of pyruvic acid when glucose was present as substrate, although the addition of insulin to the medium appeared to remedy this defect.

Inasmuch as the accumulated pyruvate is the difference between the amount formed and the amount metabolized, a change in this parameter is open to interpretation in several ways. Thus the lowered amount of accumulated pyruvic acid found after incubation of diaphragm from the E-deficient group with glucose could be the result of an increased utilization. However, since there was no concomitant increase in oxygen consumption by the diaphragm, it is unlikely that this is the correct explanation, unless the pyruvic acid was metabolized in some anaerobic transformation. There is the alternative possibility that the decreased accumulation of pyruvic acid represents a decrease in its formation from either carbohydrate or noncarbohydrate sources. Roderuck *et al.* (9) have reported that a vitamin E deficiency results in a reduction of transaminase activity. It is possible that such an effect could bring about a decreased formation of pyruvate from alanine. However, under the experimental conditions where glucose is available as substrate, it is more likely that the pyruvic acid formed by the tissues comes mainly from carbohydrate. In the latter connection it has been shown that the glucose

uptake by the tissue (which probably involves the reaction glucose to glucose-6-phosphate) was not influenced by the dietary levels of vitamin E. Similarly the glycogen formation by the tissue (which involves the series of reactions glucose-6-phosphate to glycogen) was unaffected by the level of this vitamin. This leaves the possibility that in tissue from vitamin E deficient animals, there may be a defect in the series of reactions glucose-6-phosphate to pyruvate which results in a reduced formation of pyruvic acid.

Table III shows that in these experiments, the oxygen consumption of the tissue was not affected by a vitamin E deficiency. This would appear to be in disagreement with the results reported by other investigators. Thus Friedman and Matill (3) found that semitendinous muscle taken from vitamin E deficient rats exhibited a markedly greater oxygen consumption than normal controls. Likewise Houchin and Mattill (6) found a high oxygen uptake in muscle taken from vitamin E deficient hamsters and rabbits; subsequently Houchin (5) showed that the addition of α-tocopheryl phosphate to the incubation medium lowered the oxygen consumption of such muscle slices. In all the work indicated above, however, respiration took place in a buffered medium *without added substrate* and was accordingly a measure of the endogenous metabolism of the tissue. In the experiments reported in the present investigation, the glucose in the medium served as an exogenous source of substrate. This added substrate causes relatively large increases in respiration of both normal and vitamin E deficient tissues with the result that any effect on oxygen uptake attributable to differences in vitamin status is undoubtedly masked.

In a prolonged vitamin E deficiency, a muscular dystrophy is known to occur. Although no such changes were evident in the animals in this investigation, it would appear possible from the decreased pyruvate accumulation that a biochemical lesion in muscle tissue might precede the gross pathological changes characteristic of muscular dystrophy.

It is interesting to note that the massive dose of vitamin E exerted no additional effect compared with the dose considered sufficient. Thus no change was noted in any of the parameters measured as a result of increasing the daily dose two-hundredfold. However, the plasma levels (and presumably the tissue levels) in the two groups differed only by a factor of three (1.82 and 0.66 mgm. per cent, respectively). The experimental results reported above for the two vitamin-fed groups would lead to the conclusion that for the vitamin E sensitive reactions investigated in these experiments, the systems must be close to saturation at the lower vitamin dosage.

Conclusions

The authors previously reported that vitamin E reversed the negative arteriovenous glucose differences brought about by insulin injection in the intact animal (4). This effect could be explained in part, by a possible synergism between the actions of vitamin E and insulin, resulting in an increased utilization of glucose by the peripheral tissues. The *in vitro*

experiments reported here lend little support to this hypothesis. The results suggest that tissue from vitamin E deficient animals may have a slightly decreased capacity to form pyruvic acid or else a slightly increased ability to utilize it. However, no effect on glycogen formation, glucose uptake, lactic acid accumulation, or oxygen consumption is occasioned by differences in vitamin E intake.

Although previous workers have reported that tissue from vitamin E deficient animals respires at a higher rate than normal tissue, results obtained in the present investigation suggest that this is not true when sufficient glucose is present as substrate.

Acknowledgments

The authors wish to express their appreciation to Dr. R. J. Rossiter, Head of the Department of Biochemistry, for his interest and assistance during the course of this investigation. Thanks are also due to Miss Truusje van Trigt who rendered technical assistance. Financial aid from the National Research Council of Canada and Baxter Laboratories Incorporated is gratefully acknowledged.

References

1. Barker, S. B. and Summerson, W. H. J. Biol. Chem. 138 : 535. 1941.
2. Friedemann, T. E. and Haugen, G. E. J. Biol. Chem. 147 : 415. 1943.
3. Friedman, I. and Mattill, H. A. Am. J. Physiol. 131 : 595. 1940.
4. Gray, D. E. and De Luca, H. A. Rev. can. biol. 11 : 64. 1952.
5. Houchin, O. B. J. Biol. Chem. 146 : 313. 1942.
6. Houchin, O. B. and Mattill, H. A. J. Biol. Chem. 146 : 301. 1942.
7. Morris, D. L. Science, 107 : 254. 1948.
8. Quaife, M. L., Scrimshaw, N. S., and Lowry, O. H. J. Biol. Chem. 180 : 1229. 1949.
9. Roderuck, C. E., Basinski, D. H., and Barber, M. A. Ann. N.Y. Acad. Sci. 52 : 156. 1949.
10. Stadie, W. C. and Zapp, J. A., Jr. J. Biol. Chem. 170 : 55. 1947.
11. Zierler, K. L., Levy, R. I., and Lilienthal, J. L., Jr. Bull. Johns Hopkins Hosp. 92 : 41. 1953.

THE PHARMACOLOGICAL PROPERTIES OF THE INSECTICIDE DIELDRIN[1]

By C. W. Gowdey, A. R. Graham, J. J. Seguin, and G. W. Stavraky

Abstract

The effects of dieldrin (hexachloro-epoxy-octahydro-dimethanonaphthalene) were studied in acute experiments on cats and rabbits. When injected intravenously or intra-arterially, it caused excitation of the central nervous system, which resulted in increased reflex excitability, convulsions, bradycardia, and some vasodepression. Dieldrin potentiated the effects of acetylcholine on the central nervous system and on the circulatory system as well as on intestinal motility. These latter manifestations were abolished by section of the vagus nerves, indicating a central action. Dieldrin had no effect on salivary secretion produced either by stimulation of the chorda tympani or by injections of acetylcholine in the decentralized submaxillary gland. Thus, although dieldrin has a marked parasympathomimetic action, this effect is exerted through stimulation of central mechanisms and not peripherally.

Introduction

Dieldrin (compound 497) is a new chlorinated hydrocarbon insecticide closely related chemically to aldrin. It is a white, alkali-stable, crystalline solid with the following formula: hexachloro-epoxy-octahydro-dimethanonaphthalene. Like DDT and aldrin it is insoluble in water, but is moderately soluble in the usual hydrocarbon solvents (10).

Most of the experimental studies with dieldrin have dealt with the toxic effects in laboratory and food animals and in man. Although susceptibility varies widely with species, dieldrin is said to be slightly less toxic than aldrin (2). Acute poisoning with dieldrin, as with the other chlorinated hydrocarbons, is characterized by gastrointestinal distress, vomiting, diarrhea, hyperirritability, incoordination, convulsions, coma, and death (7b). Delayed effects may occur as long as four days after oral ingestion.

In a previous report of pharmacodynamic studies with aldrin (5) we found that on injection it produced bradycardia, vasodepression, and miosis. Aldrin potentiated the effect of vagal stimulation on the heart, and increased the secretory and vasodilator effects of chorda tympani stimulation and acetylcholine injection on the salivary gland; it also increased neuromuscular transmission as tested in the sciatic–gastrocnemius preparation of the cat. Reflex excitability of the spinal cord was augmented by aldrin administration, as was the excitability of spinal centers to acetylcholine and metrazol. Repeated administration of aldrin reduced the excitability of the spinal cord and depressed neuromuscular transmission. Thus the effects of aldrin resembled in many respects those of known anticholinesterases. In the present investigation the pharmacological activity of dieldrin was studied.

Experimental Methods

Twenty-five cats and four rabbits were used in these experiments. The effects of dieldrin on the central nervous system were studied in spinal and

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Contribution from Departments of Pharmacology and Physiology, Faculty of Medicine University of Western Ontario, London, Ontario.

decerebrate cats, and in cats anesthetized with chloralose–urethane. Several different techniques have been used: namely, the quadriceps or the tibialis and gastrocnemius muscles were isolated on one or both lower extremities and recordings made from them by means of isotonic levers. The reflex activity of the spinal cord was tested by means of electrical stimulation of the central end of the peroneopopliteal or posterior tibial nerves, or by means of tapping the appropriate muscle tendons with electrically-controlled hammers. The excitability of the spinal neurones was also studied directly by means of intra-arterial injections of acetylcholine into the spinal cord.

The experiments on the circulatory system and on intestinal motility as well as those on the salivary glands were performed in animals anesthetized with a mixture of chloralose (50 mgm. per kgm.) and urethane (500 mgm. per kgm.). The effect of dieldrin on the circulatory system was studied by means of kymographic tractings of direct blood-pressure recordings (with a mercury manometer). In some experiments these recordings were combined with the measurement of the blood flow through isolated organs by means of electronic drop-recorders, or with recordings of the intestinal motility, which were done by means of a balloon inserted into the duodenum and connected to a sensitive tambour.

In the series of experiments in which the effects of dieldrin were studied on the isolated salivary gland, the animals were tracheotomized, the duct of the submaxillary salivary gland was cannulated, and the chorda tympani nerve and the cervical sympathetic nerve were severed in the neck. In these experiments the submaxillary vein was also isolated, and the blood flow through the gland was recorded.

Dieldrin was dissolved in 95% ethanol in a concentration of 40 mgm. per ml.; thus the total volume of alcohol injected was usually less than 1 ml. In an individual dose the amount of alcohol injected was usually from 0.1–0.3 ml.

Results

The effect of dieldrin on the central nervous system was studied in 20 cats of which 7 were anesthetized with chloralose and urethane, 2 were decerebrated, and 11 were made spinal. Two rabbits were also tested for convulsability.

In the anesthetized and decerebrate animals dieldrin was injected intravenously, whereas in the spinal preparations it was injected intra-arterially directly into the spinal cord by means of a plastic catheter introduced through the subclavian artery into the aorta.

In all of these experiments dieldrin produced convulsions and increased greatly the excitability of the central nervous system to reflex stimulation and to intra-arterial injections of acetylcholine.

In the cats anesthetized with chloralose and urethane intravenous injections of dieldrin in quantities from 1.9 to 7.5 mgm. per kgm. produced generalized convulsions, while in the decerebrate animals larger quantities of dieldrin (15.2 and 28 mgm. per kgm. respectively) had to be injected in order to produce this effect. In spinal preparations in which dieldrin was introduced

intra-arterially generalized twitches of the animals were also observed, but came on only after several repeated injections of dieldrin—the total amount ranging from 3.5 to 22 mgm. per kgm. In these latter experiments the convulsions were not as pronounced or as long-lasting as those seen in intact, anesthetized animals. Because of the intra-arterial route of administration of dieldrin it can be surmised that the spinal preparations were less sensitive to it than either of the two previously discussed groups of animals. Results similar to those seen in anesthetized cats were obtained in two intact unanesthetized rabbits in which dieldrin, in quantities of 1.25 to 6.0 mgm. per kgm., produced strychnine-like convulsions.

In anesthetized cats the intravenous administration of pentobarbital sodium (Nembutal) readily abolished the convulsions induced by dieldrin (Fig. 2) but they could be brought on again in the same experiments by further injections of dieldrin. It is interesting to note that acetylcholine injected intravenously also diminished or even stopped the convulsions induced by dieldrin. This effect was, however, transient (lasting up to two minutes) and was apparently associated with the hypotensive action of acetylcholine.

In Figs. 1 and 2 the effect of dieldrin on reflex stimulation of the quadriceps and of the tibialis anterior muscles, respectively, may be seen. In the first experiment (Fig. 1) the tendons of the two quadriceps muscles were tapped with electrically-driven knee-jerk hammers at a rate of 30 per minute in the spinal cat. An intra-arterial injection of 12 mgm. of dieldrin (4 mgm. per kgm.) caused, after a transient inhibition, a marked augmentation of the knee jerks, which lasted in this particular instance for four minutes, but was seen to persist over much longer periods of time on other occasions.

The transient inhibition of the contractions immediately following the injection of dieldrin was analyzed in subsequent experiments in which acetylcholine was injected as a test stimulus. This inhibition was found to be due to the alcohol in which the dieldrin was dissolved, as can be seen in tracing A and B of Fig. 3 in which a control intra-arterial injection of 95% alcohol was given in the same quantity in which it was injected with the dieldrin.

In Fig. 2 the effect of dieldrin on reflex stimulation of the tibialis anterior muscle may be observed in a cat under chloralose–urethane anesthesia. The contractions of the muscle were induced by means of electrical stimulation of the central end of the ipsilateral posterior tibialis nerve. In this experiment, as in the previous one, the injection of dieldrin in 95% ethanol, after a transient depression, caused a marked lowering of the threshold to electrical stimulation of the nerve. This was followed by a prolonged after-effect and a series of spontaneous twitches of the muscle which were completely abolished by an injection of 20 mgm. of pentobarbital sodium. A further injection of dieldrin, shown in the tracing, again brought on the spontaneous activity in the muscle, which this time could be greatly reduced by injecting 1 mgm. of pentobarbital sodium intravenously.

As mentioned previously the potentiating effect of dieldrin on the action of acetylcholine in the central nervous system may be seen in Fig. 3; the injection of 95% ethanol (Fig. 3B) served as a control.

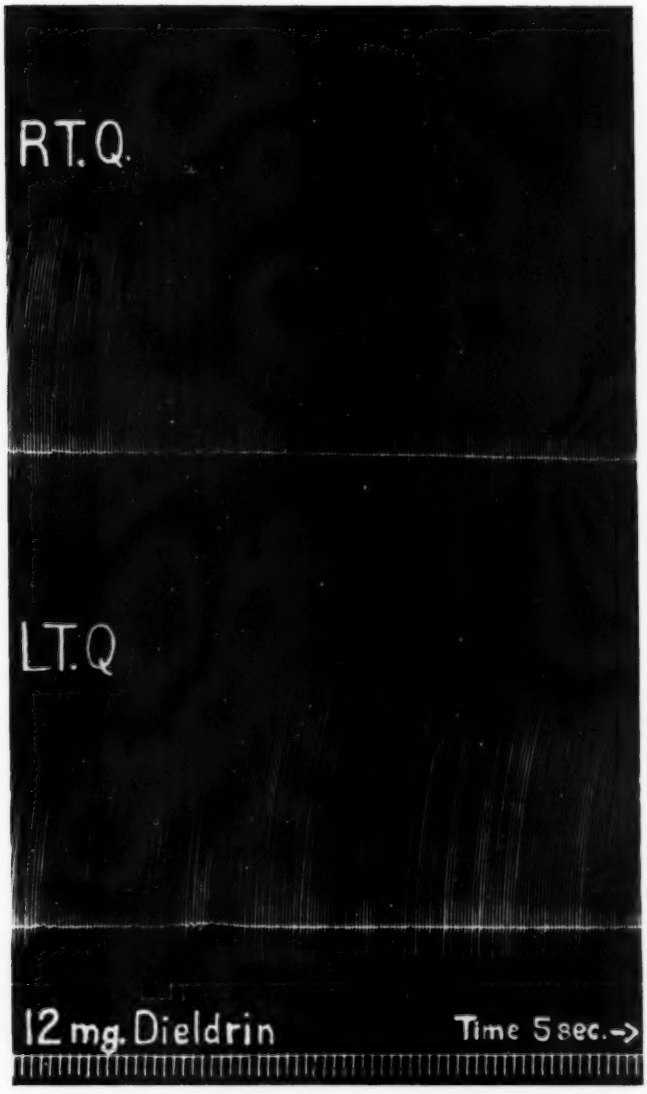

FIG. 1. Effect of dieldrin on knee jerks. Spinal cat, 2.9 kgm. Knee jerks elicited by means of bilateral tapping of patellar tendons with electrically-controlled hammers, at rate of 30/min. Note: transient depression of the knee jerks right after the injection is due to the effect of the 0.3 ml. of 95% ethanol in which the dieldrin was dissolved. (See Fig. 3.)

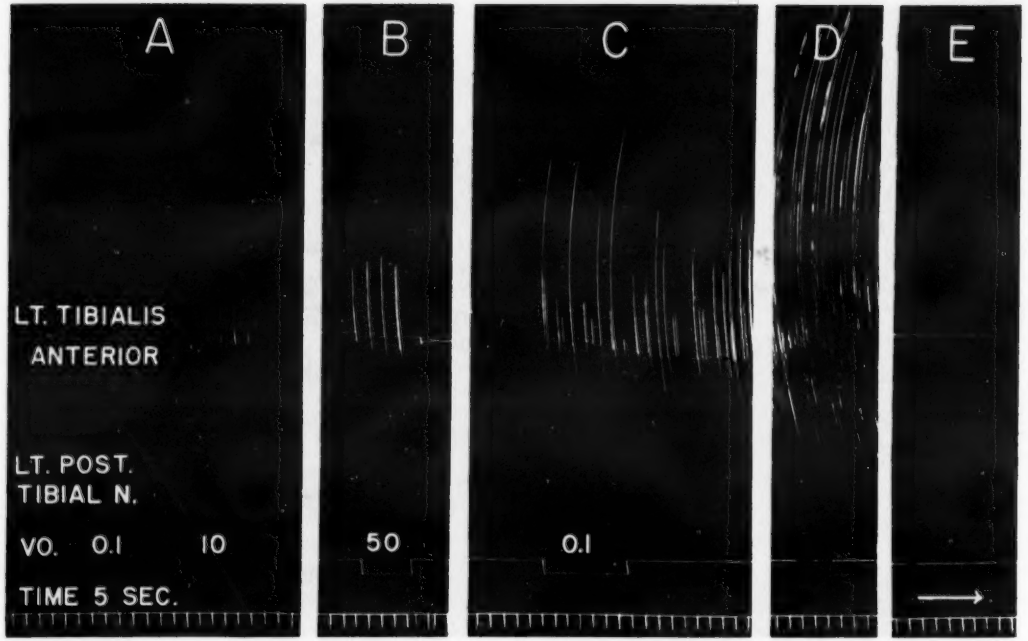

FIG. 2. Cat, chloralose–urethane anesthesia. Threshold stimulations of central end of left posterior tibial nerve at rate of 12/min. which cause reflex contractions of tibialis anterior muscle.

A. Before dieldrin. B. Three minutes after I.V. injection of 12 mgm. dieldrin (4 mgm./kgm.)—initial depression of excitability. C. Ten minutes after the injection of dieldrin—marked increase in excitability. D. Note: after-effect which lasted for eight minutes. E. When 20 mgm. Nembutal was injected I.V., the contractions stopped.

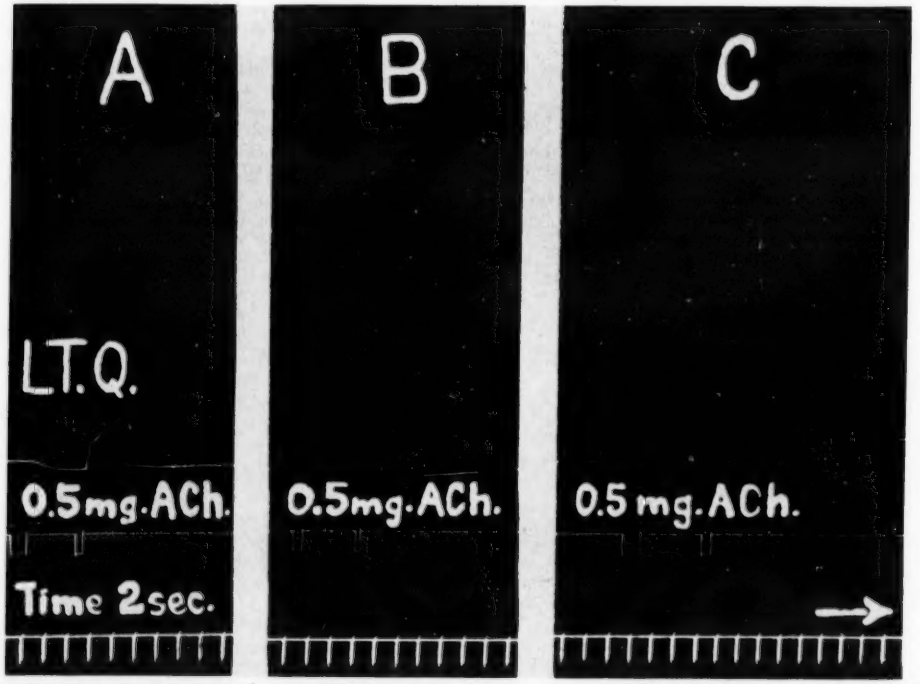

FIG. 3. Potentiation of action of acetylcholine by dieldrin on the central nervous system in the spinal cat as indicated by the contractions of the left quadriceps muscle (L.T.Q.) (all injections intra-aortally).

A. Control injection of 0.5 mgm. acetylcholine (0.17 mgm./kgm.). B. Injection of acetylcholine repeated five minutes 10 sec. after an injection of 0.2 ml. of 95% ethanol. (This quantity of ethanol equals the one in which 8 mgm. dieldrin was later injected.) Note: slightly reduced contractions of the quadriceps. Between B and C 9 mgm. (2.7 mgm./kgm.) dieldrin injected. C. Acetylcholine repeated nine minutes after dieldrin.

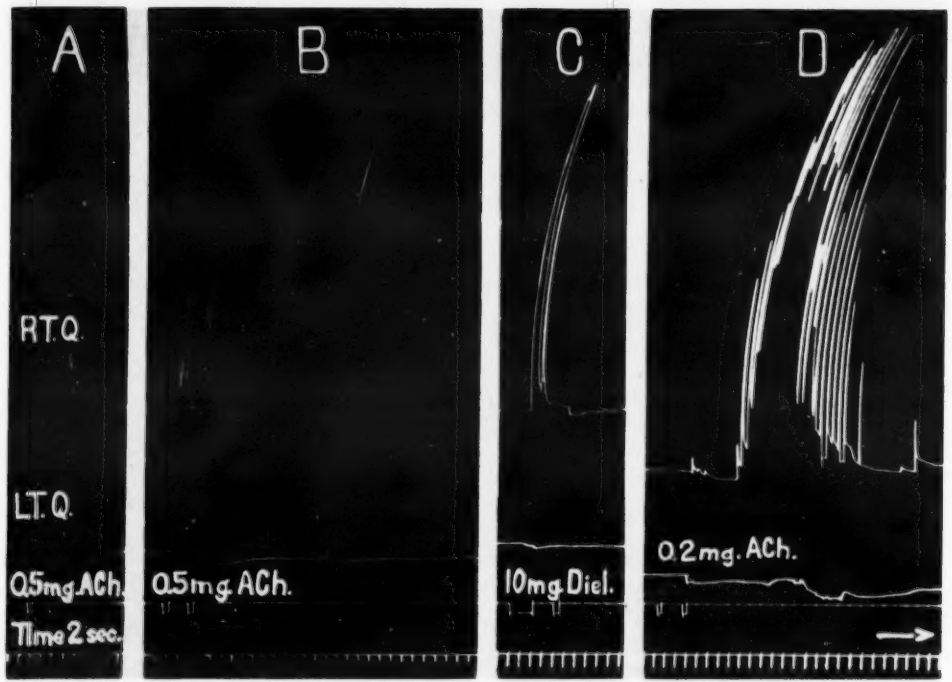

FIG. 4. Potentiation of the effect of acetylcholine on the central nervous system in the spinal cat by intra-aortal injections of dieldrin. (Right quadriceps (R.T.Q.) is innervated. Left quadriceps (L.T.Q.) was denervated by section of the femoral nerve.)

A. Control injection of acetylcholine (0.17 mgm./kgm.). After this 12 mgm. dieldrin (4.1 mgm./kgm.) were injected in divided doses. B. Marked potentiation of effect of acetylcholine on innervated quadriceps. C and D. A second injection of dieldrin produces by itself a contraction of the innervated quadriceps, and further potentiates the effect of acetylcholine in the absence of any effect on the denervated muscle.

PLATE V

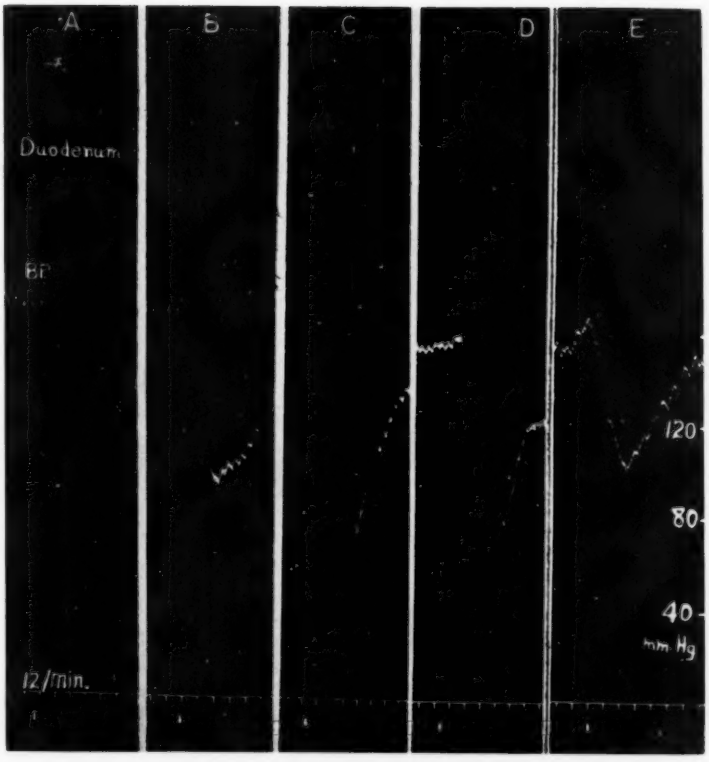

FIG. 5. Effect of dieldrin on the circulatory system and on duodenal motility in cat (under chloralose–urethane anesthesia; all injections given I.V.). In each panel the effects of an injection of 6μgm./kgm. acetylcholine are shown. Between all panels injections of 2 mgm./kgm. dieldrin were given. Before the last injection of acetylcholine the vagus nerves were sectioned in the neck. Note: progressive increase of effects of acetylcholine on the duodenal motility and on the heart; this increase was abolished by section of the vagus nerves.

PLATE VI

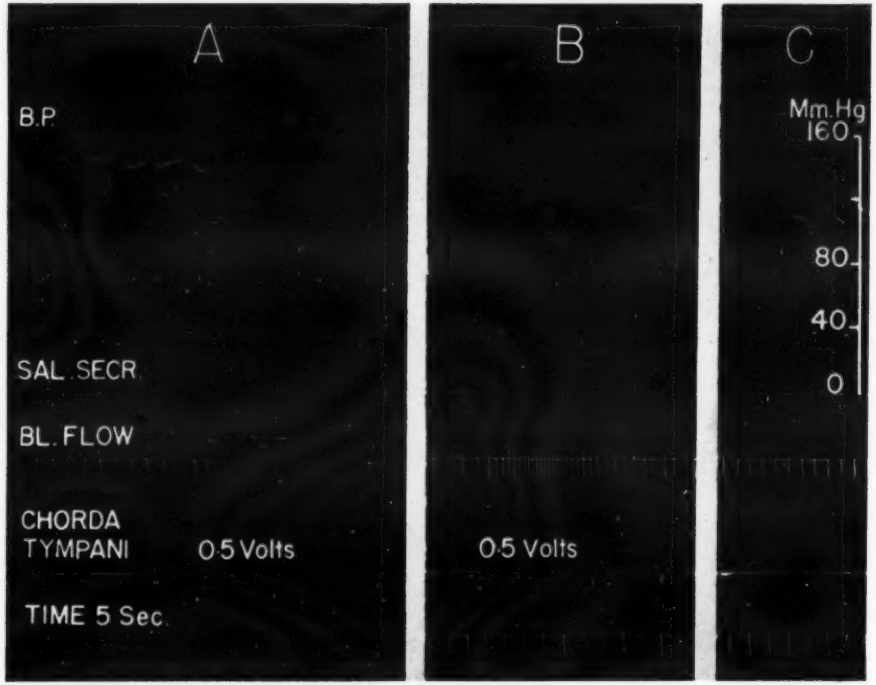

FIG. 6. Absence of any effect of dieldrin on submaxillary salivary gland in cat under chloralose–urethane anesthesia (chorda tympani and certical sympathetic trunk severed.) Between A and B 7 mgm./kgm. dieldrin injected I.V. Chorda tympani stimulated with a square-wave stimulator at frequency of 35/sec. and wave duration of 1 msec. Note: slowing of the heart after dieldrin (B) which was abolished by subsequent section of the vagus nerves (C).

The effect of dieldrin was dependent upon its action on the central nervous system (Fig. 4) and not on the muscle or other peripheral structures. An example of an experiment illustrating this is shown in Fig. 4C in which it can be seen that section of a motor nerve precluded the convulsions caused by dieldrin. Similarly the action of acethylcholine, in the quantities in which it was injected, was exerted on the spinal cord and not on the peripheral structures, and was potentiated centrally by dieldrin (Fig. 4, B and D).

The effect of dieldrin on the cardiovascular system of the cat is shown in Figs. 5 and 6. Fig. 5 shows the augmentation of the effect of acetylcholine after dieldrin on the cat's blood pressure and on duodenal motility. The depressor effect of acetylcholine was increased by dieldrin, and was accompanied by greater cardiac slowing at the time of the maximum fall in blood pressure. After several injections of dieldrin (total 7–10 mgm. per kgm.) a marked bradycardia usually developed (see Fig. 6). Bilateral section of the vagus nerves in the neck produced an immediate increase in blood pressure and heart rate. The last panel of Fig. 5 shows that the potentiation by dieldrin of the effect of acetylcholine was also abolished by vagal section. Although dieldrin itself had no effect on the movements of the duodenum, the spasm produced by acetylcholine was markedly increased by dieldrin. Bilateral vagotomy abolished this potentiation.

In a rabbit anesthetized with urethane dieldrin, injected intravenously in a dose of 1.2 mgm. per kgm., had no effect on blood pressure or respiration. An additional 2.4 mgm. per kgm., however, caused a fall of blood pressure and an increased rate and decreased depth of respiration; the respiratory rate was doubled during the period of low blood pressure. Respiration in this experiment returned to control values within 10 min., but the blood pressure did not. A further 2.4 mgm. per kgm. dieldrin caused a severe tonic convulsion and a slowing and increased depth of the respiratory movements, which finally stopped in expiration. The heart continued to beat for several minutes after cessation of respiration. The results of all four experiments of this type were comparable. In a rabbit in which intestinal motility was also recorded by a duodenal balloon it was found that dieldrin (1.25 mgm. per kgm.) abolished contractions of the duodenum at the same time as it slowed the heart and reduced the blood pressure.

Finally, in five cats under chloralose and urethane anesthesia, the effect of dieldrin was tested on the isolated submaxillary salivary gland. As seen in the tracings presented in Fig. 6, the intravenous injection of 20 mgm. of dieldrin (between tracings *A* and *B*) did not alter either the secretory effect or the vasodilatation induced by an electrical stimulation of the peripheral end of the secretory nerve to the gland. Similar experiments in which injections of acetylcholine were used instead of chorda tympani stimulation brought out the fact that dieldrin did not alter the response of the gland to parasympathetic stimulation. Neither did it have any appreciable effect on the isolated salivary gland when injected by itself.

Discussion and Conclusions

From the results presented above it may be seen that dieldrin acts predominantly on the central nervous system. Thus dieldrin caused convulsive twitches, increased the reflex excitability of the spinal cord, and also increased markedly its responses to injected acetylcholine. On the other hand, dieldrin appeared to have no effect on salivary secretion produced either by stimulation of the chorda tympani or by injection of acetylcholine in the decentralized submaxillary gland. Dieldrin itself caused bradycardia, and it increased the cardiac slowing and fall of blood pressure produced by acetylcholine when the vagi were intact. These effects were abolished by vagal section. On the small intestine dieldrin had little effect by itself, but increased somewhat the contractions elicited by acetylcholine; section of the vagus nerves prevented this augmentation.

In equivalent amounts dieldrin appears to be somewhat less potent than aldrin in its effects on the central nervous system, and is quite inactive on the peripheral nerve and striated muscle. The convulsant dose of dieldrin is larger than that of aldrin and varies widely from one animal to another; this variability is so great that experimentation with sublethal doses is often difficult. The results obtained earlier with aldrin (5) were consistent with its having a possible anticholinesterase activity: central nervous system stimulation, followed by depression; bradycardia; stimulation of the intestine; and potentiation of acetylcholine and/or nerve stimulation in the submaxillary salivary gland, the heart, the spinal centers, and the neuromuscular junction. Yet in insects aldrin was found not to have any anticholinesterase activity (11), and Crevier *et al.* (3) have reported an increase in blood serum esterase in rats after chronic poisoning with aldrin. The augmentation by dieldrin of acetylcholine responses in the heart and intestine was apparently due to stimulation of the vagal centers rather than to a peripheral action, the effect disappearing on decentralization of the various organs. Thus the main effect of dieldrin manifested itself on the central nervous system. Here it produced convulsions, augmented greatly the responses to reflex stimulation of various sensory nerves, and potentiated the effect of acetylcholine on the central nervous system. All of these effects could be overcome by the administration of barbiturates (Nembutal), and appeared again after successive injections of dieldrin. This finding is in accord with the results of Hayes (7a) who found that dieldrin-poisoned dogs and monkeys could be calmed by large doses of barbiturates.

The signs of aldrin and dieldrin poisoning resemble those following administration of DDT and are not unlike those caused by eserine. Furthermore, eserine was reported to increase DDT toxicity (2). On this account it was thought that DDT inhibited cholinesterase; Richards and Cutkomp (9), however, found no inhibition *in vitro* of esterase activity. Tobias, Kollros, and Savit (12a) felt that the failure of DDT to inhibit cholinesterase *in vitro* was of questionable significance because of its extreme insolubility in water. They found during the period of prostration of the insect following DDT that

the acetylcholine content in the central nervous system of the cockroach and fly increased markedly, but it did not change in the rat or frog. Brown (2) makes the point that the central nervous system of the insect is physiologically different from that of the mammal; it closely resembles the parasympathetic system of vertebrates. Tobias *et al.* (12a) stated that to their knowledge DDT administration was the only *in vivo* condition aside from the injection of potassium ions which increased the acetylcholine content of a structure composed primarily of nerve fibers by a non-anticholinesterase. They suggested that DDT might liberate acetylcholine from a bound ester form—which is thought to be an acetylcholine–lipoprotein complex. Others (1, 6a, b, 12a, b, 13, 14, 15) also reported that the cholinesterase activity appeared to be unchanged in various tissues (brain, peripheral nerve, salivary glands, and blood serum) of various animals (frog, rat, mouse, rabbit, dog, sheep, horse, and monkey) after DDT poisoning. In insects especially, an increase in acetylcholine content was found (6b, 12a, b, 15) in the last stage of poisoning, but Dresden (4) thought that this increase was the result of nervous hyperactivity rather than its cause. According to Philips and Gilman (8) DDT has a central action on the autonomic nervous system in mammals. Our results with dieldrin before and after section of the vagus nerves support this hypothesis. Whether the predominant site of action of the insecticide will be on the autonomic or on the somatic nervous system will depend upon their specific susceptibilities to the agent.

References

1. Borecker, B. L. and Sambursky, G. G. Insect Control Comm., Washington, Abstr. on Insect & Rodent Control, No. 2, CC-9-334. 1946; quoted from (4).
2. Brown, A. W. A. Insect control by chemicals. John Wiley & Sons, Inc., New York. 1951.
3. Crevier, M., Monkman, J. L., and Ball, W. L. Proc. Can. Physiol. Soc. 16 : 19. 1952.
4. Dresden, D. Physiological investigations into the action of DDT. G. W. Van der Wiel & Co., Arnhem. 1949.
5. Gowdey, C. W., Graham, A. R., Seguin, J. J., Stavraky, G. W., and Waud, R. A. Can. J. Med. Sci. 30 : 520. 1952.

6a. Guzman Barron, E. S. Insect Control Comm., Washington, Abstr. on Insect & Rodent Control, No. 3, CC-14-1. 1946; quoted from (4).

b. Guzman Barron, E. S. Insect Control Comm., Washington, Abstr. on Insect & Rodent Control, No. 3, CC-14-3, 4. 1946.

7a. Hayes, W. J. Food and Drug Admin. Hearings on Residue Tolerances, Exhibit 1212. 1950.

b. Hayes, W. J., Ferguson, F. F., and Cass, J. S. Food and Drug Admin. Hearings on Residue Tolerances, Exhibit 1211. 1950.

8. Philips, F. S. and Gilman, A. Z. J. Pharmacol. Exptl. Therap. 86 : 213, 222. 1946.
9. Richards, A. G. and Cutkomp, L. K. J. Cellular Comp. Physiol. 26 : 57. 1945.
10. Shell Chemical Corp. Entomol. Rept.—Dieldrin (Compound 497). New York. 1950.
11. Smallman, B. N. Dominion of Can. Science Service Lab., London. Personal communication.

12a. Tobias, J. M., Kollross, J. J., and Savit, J. J. Cellular Comp. Physiol. 28 : 159. 1946.

b. Tobias, J. M., Kollross, J. J., and Savit, J. Insect Control Comm., Washington, Abstr. on Insect & Rodent Control, No. 5, CC-9-624. 1946; quoted from (4).

13. Truhart, R. and Vincent, D. Am. pharm. franç. 5 : 159. 1947; quoted from Chem. Abstr. 42 : 680. 1948.
14. Vincent, D., Truhart, R., and Abadie, A. Compt. rend. soc. biol. 142 : 1500. 1948.
15. Welsh, J. H. Insect Control Comm., Washington, Abstr. on Insect & Rodent Control, No. 3, CC-17-3. 1946; quoted from (4). No. 5, CC-17-1, 4, 7. 1946.

INCORPORATION OF RADIOACTIVE PHOSPHATE INTO NON-NUCLEOTIDE PROTEIN-BOUND PHOSPHORUS FRACTIONS OF RESPIRING CAT BRAIN SLICES[1]

By Muriel Findlay, K. P. Strickland[2], and R. J. Rossiter

Abstract

Slices of cat brain respiring in a Krebs–Ringer bicarbonate medium were found to incorporate inorganic phosphate labelled with P^{32} into two non-nucleotide protein-bound phosphorus fractions, which are referred to as the residue organic phosphorus (ROP) and the "phosphoprotein" (PP), respectively. The addition of glucose or mannose to the medium increased the incorporation into both fractions. The addition of fructose, galactose, pyruvate, lactate, succinate, or L-glutamate failed to increase the incorporation into either fraction.

Anaerobic conditions, homogenization of the tissue, or the addition of a wide range of metabolic inhibitors (cyanide, azide, iodoacetate, fluoride, nembutal, malononitrile, chloretone) inhibited the incorporation. The incorporation was also inhibited by 2,4-dinitrophenol in concentrations that do not inhibit oxygen consumption.

These results are compared and contrasted with previous findings on the incorporation of P^{32} into the lipid phosphorus and pentosenucleic acid of brain slices.

Introduction

It is widely believed that the greater part of the phosphorus that remains in the protein residue of animal tissues after the removal of the acid-soluble and lipid phosphorus is present in the form of nucleotide phosphorus. The phosphorus may be in either pentosenucleic acid (PNA) or desoxypentosenucleic acid (DNA). With brain tissue, Logan, Mannell, and Rossiter (17) reported that more than one-half of the protein-bound phosphorus remaining in the tissue residue after the removal of the acid-soluble and lipid phosphorus by the usual procedures was not accounted for by the phosphorus of the nucleotides. Most of this non-nucleotide phosphorus remains in the tissue residue after the nucleic acids are extracted by the method of Hammarsten (11). The phosphorus of this residue can be separated by alkaline hydrolysis into an inorganic fraction, thought to be derived from "phosphoproteins" (referred to as PP) and an organic fraction. Previously (17), this fraction was called "inositide P". In the present publication it will be referred to as the residue organic phosphorus (ROP).

DeLuca, Rossiter, and Strickland (5) reported that if slices of cat brain were incubated in a Krebs–Ringer bicarbonate buffer containing glucose and inorganic phosphate labelled with P^{32}, some of the P^{32} was incorporated into the PNA of the slice. Findlay, Rossiter, and Strickland (8) showed that this incorporation was sustained by the addition of glucose, mannose, pyruvate, or lactate, but not by the addition of succinate, L-glutamate, or certain of the tricarboxylic acid cycle intermediates. The incorporation was inhibited by

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anaerobic conditions, the addition of a number of metabolic inhibitors, or by the presence of 2,4-dinitrophenol in concentrations that did not decrease the oxygen consumption. It was concluded that the incorporation was dependent upon oxidative phosphorylating mechanisms within the slice.

Fries, Schachner, and Chaikoff (10) and Schachner, Fries, and Chaikoff (28) showed that inorganic P^{32} was incorporated into the lipid P of respiring slices of rat brain. Strickland (32) confirmed the findings for cat brain and showed that the conditions necessary for the incorporation were similar to those described by Findlay *et al.* (8) for the incorporation of inorganic P^{32} into PNA.

During the course of the experiments with PNA it became clear that considerable quantities of P^{32} were being incorporated into the PP and ROP fractions. Because of this incorporation of P^{32} into non-nucleotide P, the PNA nucleotides, as obtained by the method of Schmidt and Thannhauser (29), had an apparent specific activity many times greater than their true specific activity (5). In this paper the factors that affect the incorporation of P^{32} into the ROP and PP, i.e. the non-nucleotide P fractions, are described for respiring slices of cat brain. These are compared with the factors that affect the incorporation of P^{32} into the PNA and lipid P.

Methods

Slices of cat brain were incubated in Warburg flasks containing Krebs–Ringer bicarbonate, glucose, and radioactive phosphate* (P^{32}). The vessels were filled with a mixture of 95% oxygen and 5% carbon dioxide and incubated in a bath at 37.2° C. for four hours with shaking to ensure proper oxygenation. Details of the preparation of the tissue and the incubation have been described previously (5). In some experiments glucose was omitted from the incubating medium or replaced by other substrates. Solutions of substrates and inhibitors were freshly prepared in Krebs–Ringer bicarbonate. Where necessary, the solution was titrated to the pH of the buffer with hydrochloric acid or sodium hydroxide. For anaerobic experiments the flasks were filled with a mixture of 95% nitrogen and 5% carbon dioxide which had been passed over heated copper filings to remove the last traces of oxygen.

After the incubation period the tissue was homogenized and the acid-soluble P, containing the greater part of the radioactivity, was removed as described by DeLuca *et al.* (5). The lipid P was extracted and purified as described by Strickland (31, 32). The nucleic acids were then removed from the protein residue by the method of Hammarsten (11) and the PNA nucleotides were further purified by two magnesia treatments. For brain slices the mixed nucleotides so obtained have a specific activity within the range given by the individidual nucleotides separated by paper chromatography (5).

The residue remaining after the extraction of the nucleic acids by the method of Hammarsten (11) was extracted with 5 ml. 5% trichloroacetic acid (TCA) at 90° C. for 15 min. followed by a wash with 5% TCA. This

* *Radioactive phosphate was received in the form of* $H_3P^{32}O_4$ *from the Eldorado Mining and Refining (1944) Limited.*

treatment removed the last traces of nucleic acids. The residue was then hydrolyzed in *N* potassium hydroxide as described by Schmidt and Thannhauser (29). On acidification a precipitate appeared, which contained a small amount of phosphorus of very low specific activity. This fraction was discarded. The inorganic P of the acid-soluble portion of the hydrolyzate is derived from the hydrolysis of "phosphoprotein". It forms the PP. The organic P of the hydrolyzate, after two further magnesia treatments, forms the residue organic P (ROP).

The specific activity of the PP was determined by the method of Ernster, Zetterström, and Lindberg (7), as were the specific activities of the purified lipid P, PNA nucleotides, and ROP, after ashing with perchloric acid. In this method both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue color complex was read with a Beckman Model B spectrophotometer at 730 mμ, after which the radioactivity due to the P^{32} in the sample was determined on the colored solution by the use of an M-6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay and the specific activities were calculated on the basis of a standard count of 8.1×10^5 impulses/min./ml. incubating medium.

Results

Glucose as Substrate

Table I shows the results of four different experiments that demonstrate the incorporation of P^{32} into the ROP and PP of the slices of cat brain. The low specific activities at zero hours indicate that very little of the activity found

TABLE I

SPECIFIC ACTIVITY OF THE ROP AND PP OF SLICES OF CAT BRAIN INCUBATED IN MEDIUM CONTAINING P^{32}

(counts/min./μgm. P)

		Expt. 1	Expt. 2	Expt. 3	Expt. 4
ROP	Zero hours Glucose 0.011 *M*	1.3	0.3	0.7	4.4
	Four hours Glucose 0.011 *M*	201	140	178	214
	Four hours No glucose	54	38	36	68
PP	Zero hours Glucose 0.011 *M*	25	30	36	45
	Four hours Glucose 0.011 *M*	800	500	1010	920
	Four hours No glucose	450	330	580	540

in the four hour slices could be due to contamination of the fraction with inorganic P^{32}, since the zero hour slices were suspended in the medium containing the same amount of P^{32} as the incubated samples and the homogenization was carried out in the presence of the P^{32}. It can be seen from Table I that when glucose, which is known to increase the oxygen consumption of brain slices, was omitted from the medium there was a decrease in the incorporation of P^{32} into both the ROP and the PP, the decrease in the incorporation into the ROP being relatively much greater. These findings are similar to those reported previously for PNA and lipid P (8, 32).

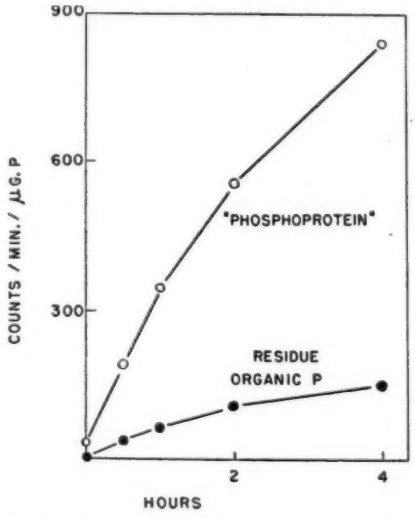

FIG. 1. Time course of the incorporation of P^{32} into the residue organic P (ROP, ●—●—●) and "phosphoprotein" (PP ○—○—○) of slices of cat brain respiring in medium containing P^{32}.

Fig. 1 shows the time course of the incorporation of P^{32} into the ROP and PP. As previously found for lipid P, the rate of increase of specific activity fell off after the first hour (32). This is in contrast to the findings for PNA, where the curve was linear with time for six hours (8).

As found previously for both PNA and lipid P, the incorporation of P^{32} into the ROP and PP was almost completely abolished by anaerobic conditions, or by homogenizing the tissue.

Table II presents the results of two experiments in which, in addition to the specific activity, the concentration of each fraction was determined by the method of Logan, Mannell, and Rossiter (17). From these data was calculated the activity of each fraction in terms of the number of impulses per minute given by 100 mgm. wet weight of brain. In each experiment the order of the specific activities was: PP> ROP> PNA> lipid P. In terms

of the specific activity of the inorganic P of the medium, these figures were approximately 4%, 0.7%, 0.1%, and 0.05%, respectively. For 100 mgm. of brain tissue, similar amounts of P^{32} were incorporated into the lipid P, ROP, and PP fractions. Although the concentration of PP was low compared with that of the lipid P, the specific activity was much greater, so that the amount of P^{32} incorporated into 100 mgm. brain was of the same order for each of these fractions. The amount of P^{32} incorporated into the PNA was only 10% of that incorporated into each of the other three fractions.

TABLE II

SPECIFIC ACTIVITY AND ACTIVITY PER 100 MGM. TISSUE OF THE LIPID P, PNA, ROP, AND PP OF SLICES OF CAT BRAIN INCUBATED IN MEDIUM CONTAINING P^{32}

Fraction		Concentration (μgm.P/100 mgm. tissue)	Specific activity (counts/min./mgm. P)	Activity (counts/min./100 mgm. tissue)
Expt. 1	Lipid P	230	9.8	2250
	PNA	10.5	19.2	201
	ROP	13.0	151	1960
	PP	3.0	840	2520
Expt. 2	Lipid P	210	12.6	2650
	PNA	8.2	24.5	201
	ROP	12.9	147	1900
	PP	2.9	610	1770

Other Substrates

The effects of substituting each of a number of other substrates for glucose are summarized in Table III, which shows data for lipid P, PNA, ROP, and PP. Although each of the substrates tested is known to increase the oxygen consumption of brain slices (15, 16, 26, 36), only glucose and mannose caused a significant increase in the incorporation of P^{32} into all of the fractions. Galactose, L-glutamate, and succinate were without a significant effect on any fraction, while fructose, pyruvate, and lactate varied somewhat in their effect from fraction to fraction.

Table III shows that for any given substrate the extent of the increase in the incorporation differed from fraction to fraction. The percentage increase after the addition of glucose was in the order: ROP> lipid P> PP> PNA. Despite these differences between fractions, however, there was some unity in the substrate effect in that certain substrates were effective, whereas others were ineffective, for all fractions. In addition, for substrates that were effective in stimulating the incorporation, the percentage increase for each substrate was usually in the same order as that reported above for glucose.

TABLE III

EFFECT OF DIFFERENT SUBSTRATES ON THE MEAN INCREASE IN THE SPECIFIC ACTIVITY OF THE LIPID P, PNA, ROP, AND PP OF SLICES OF CAT BRAIN INCUBATED IN MEDIUM CONTAINING P^{32}

(Mean percentage increase ± S.E.M., the increase being relative to the specific activity with no added substrate. Incubation time, four hours. Number of determinations in parentheses)

Substrate	Concentration (*M*)	Lipid P	PNA	ROP	PP
Glucose	0.011	194 ± 13 (8)	38 ± 6 (6)	285 ± 23 (8)	82 ± 16 (8)
Mannose	0.01	192 ± 26 (4)	25 ± 11 (3)	308 ± 15 (4)	62 ± 16 (4)
Fructose	0.01	58 ± 7 (4)	1 ± 15 (3)	45 ± 12 (4)	23 ± 11 (4)
Galactose	0.01	1 ± 4 (4)	*14 (33, −6)	13 ± 14 (4)	−3 ± 5 (4)
Pyruvate	0.01	30 ± 5 (3)	*52 (82, 22)	38 ± 8 (3)	12 ± 9 (3)
Lactate	0.01	25 ± 9 (4)	16 ± 0 (3)	19 ± 24 (4)	13 ± 18 (4)
L (+)-glutamate	0.01	1 ± 3 (4)	−5 ± 2 (4)	−11 ± 4 (4)	−4 ± 15 (4)
*Succinate	0.05	−10 (−9, −10)	−13 (6, −31)	−16 (−12, −20)	−11 (−17, −5)

* *Mean of the two values listed in brackets.*

Inhibitors

Table IV shows the effect of a number of metabolic inhibitors on the incorporation of P^{32} into the lipid P, PNA, ROP, or PP fractions. For the most part the inhibitors were used in concentrations that depressed the respiration. It can be seen from the table that, in general, for any concentration of a given inhibitor, there was a remarkable similarity in the degree of inhibition found for the incorporation into each of the fractions. This is well illustrated by the experiments with the lower concentrations of malononitrile and chloretone.

A careful examination of the data of Table IV shows that the percentage inhibition produced by a given concentration of inhibitor was usually closer for the lipid P, PNA, and ROP fractions than for the PP. The PP was an exception in that the percentage inhibition was usually somewhat less than the value observed for the other fractions.

2,4-Dinitrophenol (DNP)

DNP is representative of a class of metabolic inhibitors that inhibit aerobic phosphorylation without causing a corresponding decrease in oxygen consumption. In many tissue preparations the oxygen consumption is increased by concentrations of DNP effective in inhibiting phosphorylation.

TABLE IV

INHIBITION OF THE INCORPORATION OF P^{32} INTO THE LIPID P, PNA, ROP, AND PP OF SLICES OF CAT BRAIN

(Decrease in the specific activity in the presence of the inhibitor, expressed as a percentage of of the uninhibited control. Incubation time, four hours)

Inhibitor	Concentration (M)	Lipid P	PNA	ROP	PP
Cyanide	3×10^{-2}	94	94	95	87
Azide	10^{-2}	97	99	97	90
Indoacetate	10^{-3}	87	67	89	78
Fluoride	10^{-2}	85	67	89	54
Nembutal	10^{-3}	57	48	56	45
Malononitrile	10^{-2}	83	94	80	71
	10^{-3}	20	42	18	18
	10^{-4}	0	7	0	4
Chloretone	4×10^{-3}	73	79	74	52
	2×10^{-3}	35	44	31	26
	10^{-3}	21	25	18	7
Nitrogen gas phase	—	92	90	94	87

TABLE V

EFFECT OF DNP ON INCORPORATION OF P^{32} INTO THE LIPID P, PNA, ROP, AND PP OF SLICES OF CAT BRAIN

(Decrease in the specific activity in the presence of the inhibitor, expressed as a percentage of the uninhibited control)

	Conc. DNP (M)	Incubation time (hr.)	Lipid P	PNA	ROP	PP
Expt. 1	10^{-3}	4	95	93	97	91
	10^{-4}		93	83	94	83
	10^{-5}		20	2	20	3
Expt. 2	10^{-4}	4	79	52	77	61
	7.5×10^{-5}		71	50	70	53
	5×10^{-5}		53	29	46	40
	2.5×10^{-5}		38	11	40	29
	10^{-5}		16	9	11	7
Expt. 3	5×10^{-5}	0.5	54	58	50	45
		4	69	58	67	52

Table V shows the results of some representative experiments with DNP. Experiment 1 demonstrated the inhibitory effect of DNP on the incorporation of P^{32} into each of the fractions. DNP, in a concentration of 10^{-3} *M*, decreases the oxygen consumption of brain slices, but concentrations in the range 10^{-4}–10^{-5} *M* increase the oxygen consumption (21, 24, 34). Experiment 2 shows the effect of these concentrations of DNP on the incorporation of P^{32} into each of the fractions.

Experiments 1 and 2 show that for PNA the inhibitory effect of DNP was usually less than the effect found for the lipid P and ROP fractions. This decrease in the percentage inhibition for PNA was quite consistent under the conditions of the experiment. However, Experiment 3 shows that, if the incubation time was reduced from 4 hr. to 0.5 hr., the inhibition for PNA was no longer less than that for lipid P and ROP. This experiment, which was repeated several times, revealed that for lipid P, ROP, and PP, the percentage inhibition brought about by the DNP increased with the time of incubation, whereas for PNA the extent of the inhibition was the same for all time intervals studied.

Discussion

The results indicate that P^{32} is incorporated *in vitro* into the ROP and PP of brain slices and that the incorporation is dependent upon the metabolism of the slice. The presence of a wide range of metabolic inhibitors, the absence of oxygen, or fragmentation of the tissue, greatly decreases the incorporation. As previously discussed for PNA and lipid P, the necessary prerequisites for maximum incorporation are: aerobic conditions, the presence of an energy-yielding substrate, and the maintenance of adequate concentrations of phosphocreatine and other labile P compounds (8, 32). It should be stressed that during the period of incubation there is no demonstrable net synthesis of any of the fractions, since the total quantity of each fraction in the slice remains the same.

It is of interest to note that L-glutamate, a substrate that increases the oxygen consumption of brain slices, failed to support the incorporation of P^{32} into lipid P (32), PNA (8), ROP, or PP (6). In the presence of glutamate, slices of guinea pig brain did not maintain their level of phosphocreatine (19). Glutamate abolished the usual metabolic response of guinea pig brain slices to *in vitro* stimulation, but was without effect on slices of human brain (20). Weil-Malherbe (37, 38) has summarized the evidence for the view that L-glutamate is not an energy-yielding substrate for brain tissue. In experimental animals this may apply to brain slices and also to brain metabolism *in vivo* for, unlike glucose or mannose, glutamate was unable to abolish the changes in the electroencephalogram produced by the progressive hypoglycemia of hepatectomized animals (22), nor was it able to support the respiration of perfused cat brain (35).

Succinate, like glutamate, failed to support the incorporation of P^{32} into lipid P and PNA (8, 32). In the presence of succinate, slices of guinea pig

brain also failed to maintain their levels of phosphocreatine and adenosine triphosphate (14). In slices of both human and guinea pig brain there was no metabolic response to *in vitro* stimulation when succinate was the substrate (14, 20). It is probable that succinate also is not an energy-yielding substrate for brain slices, for it failed to support either the formation of acetylcholine (25) or the active transport of glutamate into brain slices against a concentration gradient (30). *In vivo*, succinate failed to prevent the changes in the electroencephalogram produced by progressive hypoglycemia (22) and also failed to maintain the functional activity of the perfused spinal cord of the rat (33).

Thus with experimental animals neither glutamate nor succinate appears to be a useful substrate for *in vivo* oxidation by brain tissue or for *in vitro* oxidation in brain slices. This is of some interest, for the experiments of Abood and Gerard (1) and Brody and Bain (2) show that with particulate preparations of brain tissue both glutamate and succinate are able to support oxidative phosphorylation. Both substrates also support the incorporation of P^{32} into the adenosine polyphosphates (1).

The conditions that support or inhibit the incorporation of P^{32} into the ROP and PP of brain slices are similar to those that support or inhibit the incorporation of P^{32} into lipid P (32) or PNA (8). The parallel between the conditions that support P^{32} incorporation and the conditions, studied by McIlwain and his colleagues (14, 19), that enable slices of brain tissue to maintain their level of phosphocreatine has been commented upon previously (8, 32). The conditions for optimal incorporation into each of the fractions are so similar that the possibility must be considered seriously that all fractions possess some factor in common, possibly the incorporation of P^{32} into a shared acid-soluble precursor. Despite the similar pattern for all four fractions, there are some notable differences, especially for the PNA fraction. These include: (1) the fraction-to-fraction differences in the percentage stimulation brought about by glucose and other metabolizable substrates (Table III), (2) the difference in the time course of the incorporation for PNA, and (3) the difference in the extent and in the time course of the inhibition of the incorporation of P^{32} into PNA brought about by DNP. Such findings suggest that some of the enzymes beyond the initial phosphorylating step may vary in their stability.

Concerning the chemical nature of ROP and PP, little can be said at present. Logan *et al.* (17) referred to the ROP as "inositide P". It may be, in part, derived from the inositol-containing complex isolated from brain tissue by Folch and LeBaron (9). This fraction, which occurs in higher concentration in white matter than in gray matter of brain (17), disappears from a peripheral nerve as the nerve degenerates (18). It may therefore be related to the myelin sheath of the nerve fiber. The fraction is analogous to the "concomitant P" described by Davidson and Smellie (4) for liver tissue. Middleton (23) has shown that this fraction, like the concomitant P of liver tissue, comprises a number of metabolically active P-containing compounds.

The precise nature of the PP of brain tissue is also unknown. The specific activity of this fraction was considerably greater than that of any of the other protein-bound P compounds. Similar findings were previously reported for *in vivo* experiments with brain (31) and liver (3, 4, 13). The fact that there was no incorporation into the PP in the presence of metabolic inhibitors, under anaerobic conditions, and at zero time, is convincing evidence that the high specific activity of this fraction is due to a genuine metabolic incorporation of the P^{32}. In these experiments all the manipulations were carried out in the presence of the usual concentrations of P^{32}. The experiments would appear to rule out the possibility that the high specific activity of the PP is the result of a nonspecific adsorption of highly active inorganic P by the proteins at the time they were precipitated with TCA.

The results presented here are in agreement with a preliminary report of Engelhardt and Lissovskaia (6). These workers found that the absence of oxygen or glucose, or the addition of cyanide, azide, DNP, or glutamic acid, caused a great reduction in the incorporation of P^{32} into the PP of brain slices. They also reported that glucose was the only substrate that supported the incorporation of P^{32} into the PP. Table III shows that for the PP fraction, unlike some of the other fractions, fructose, galactose, pyruvate, and lactate were without significant effect, but that mannose was almost as effective as glucose.

As has been suggested already (6, 13), it is possible that the "phosphoproteins", with their high specific activity, may represent enzyme–phosphate complexes. Although it is thought unlikely that an enzyme–phosphate complex of this type is formed by glyceraldehyde-3-phosphate dehydrogenase at the stage where inorganic P first enters the metabolic pathway (27), such complexes are presumably quite general for phosphate-transferring enzymes, such as the phosphomutases* and the phosphokinases (ADP-transphosphorylases).

The high specific activity of the ROP and PP fractions and the high concentration of the ROP fraction in brain tissue again emphasizes previous statements that the method of Schmidt and Thannhauser (29) is not suited to the determination of the specific activities of RNA (3, 4, 5, 31).

References

1. Abood, L. G. and Gerard, R. W. Am. J. Physiol. 168 : 739. 1952.
2. Brody, T. M. and Bain, J. A. J. Biol. Chem. 195 : 685. 1952.
3. Davidson, J. N., Frazer, S. C., and Hutchinson, W. C. Biochem. J. (London), 49 : 311. 1951.
4. Davidson, J. N. and Smellie, R. M. S. Biochem. J. (London), 52 : 599. 1952.
5. DeLuca, H. A., Rossiter, R. J., and Strickland, K. P. Biochem. J. (London), 55 : 193. 1953.
6. Engelhardt, V. A. and Lissovskaia, N. P. *In* Communication at the XIX International Physiological Congress. Acad. Sci. U.S.S.R. Moscow. 1953. p. 207.
7. Ernster, L., Zetterström, R., and Lindberg, O. Acta Chem. Scand. 4 : 942. 1950.

* *Jagannathan and Luck (12) have shown that the enzyme phosphoglucomutase contains nondialyzable P that becomes radioactive when the enzyme acts on substrates labelled with* P^{32}.

8. FINDLAY, M., ROSSITER, R. J., and STRICKLAND, K. P. Biochem. J. (London), 55 : 200. 1953.
9. FOLCH, J. and LE BARON, F. M. Federation Proc. 10 : 183. 1951.
10. FRIES, B. A., SCHACHNER, H., and CHAIKOFF, I. L. J. Biol. Chem. 144 : 59. 1942.
11. HAMMARSTEN, E. Acta Med. Scand. Suppl. No. 196, 128 : 634. 1947.
12. JAGANNATHAN, V. and LUCK, J. M. J. Biol. Chem. 179 : 569. 1949.
13. JOHNSON, R. M. and ALBERT, S. J. Biol. Chem. 200 : 335. 1953.
14. KRATZING, C. C. Biochem. J. (London), 54 : 312. 1953.
15. KREBS, H. A. Biochem. J. (London), 29 : 1620. 1935.
16. LOEBEL, R. O. Biochem. Z. 161 : 219. 1925.
17. LOGAN, J. E., MANNELL, W. A., and ROSSITER, R. J. Biochem. J. (London), 51 : 470. 1952.
18. LOGAN, J. E., MANNELL, W. A., and ROSSITER, R. J. Biochem. J. (London), 51 : 482. 1952.
19. MCILWAIN, H. J. Mental Sci. 97 : 674. 1951.
20. MCILWAIN, H. J. Neurol. Neurosurg. Psychiat. 16 : 257. 1953.
21. MCILWAIN, H. and GORE, M. B. R. Biochem. J. (London), 50 : 24. 1951.
22. MADDOCK, S., HAWKINS, J. E., and HOLMES, E. Am. J. Physiol. 125 : 551. 1939.
23. MIDDLETON, E. J. To be published.
24. PEISS, C. N. and FIELD, J. J. Biol. Chem. 175 : 49. 1948.
25. QUASTEL, J. H., TENNEBAUM, M., and WHEATLEY, A. H. M. Biochem. J. (London), 30 : 1668. 1936.
26. QUASTEL, J. H. and WHEATLEY, A. H. M. Biochem J. (London), 26 : 725. 1932.
27. RACKER, E. and KRIMSKY, I. J. Biol. Chem. 198 : 731. 1952.
28. SCHACHNER, H., FRIES, B. A., and CHAIKOFF, I. L. J. Biol. Chem. 146 : 95. 1942.
29. SCHMIDT, G. and THANNHAUSER, S. J. J. Biol. Chem. 161 : 83. 1945.
30. STERN, J. R., EGGLESTON, L. V., HEMS, R., and KREBS, H. A. Biochem. J. (London), 44 : 410. 1949.
31. STRICKLAND, K. P. Can. J. Med. Sci. 30 : 484. 1952.
32. STRICKLAND, K. P. Can. J. Biochem. Physiol. 32 : 50. 1954.
33. TSCHIRGI, R. D., GERARD, R. W., JENERICK, H., BOYARSKY, L. L., and HEARON, J. Z. Federation Proc. 8 : 166. 1949.
34. TYLER, D. B. J. Biol. Chem. 184 : 711. 1950.
35. WAELSCH, H. Advances in Protein Chem. 6 : 299. 1951.
36. WEIL-MALHERBE, H. Biochem. J. (London), 30 : 665. 1936.
37. WEIL-MALHERBE, H. Physiol. Revs. 30 : 549. 1950.
38. WEIL-MALHERBE, H. Biochem. Soc. Symposia (Cambridge, Engl.), No. 8 : 16. 1952.

ENZYMATIC DECARBOXYLATION OF 2,6- AND 2,3-DIHYDROXYPHENYLALANINE[1]

By Theodore L. Sourkes

Abstract

The action of dihydroxyphenylalanine (dopa) decarboxylase of guinea pig kidney and of tyrosine decarboxylase of *Streptococcus faecalis R* on 2,3- and 2,6-dopa has been studied. Compared with 3,4-dopa, these substrates are decarboxylated by the kidney enzyme at rates decreasing in the order: 2,3-, 3,4-, 2,6-dopa. The bacterial enzyme employed as an acetonized powder decarboxylates 2,3-dopa but not the di-ortho isomer. The significance of these results in clarifying the substrate specificity of the two enzymes is discussed.

Introduction

The enzymatic decarboxylation of mono- and di-hydroxy derivatives of phenylalanine has been the subject of a number of reports in recent years. Two types of enzyme catalyze such reactions, namely, dihydroxyphenylalanine (dopa) decarboxylase of mammalian tissues (4) and bacterial tyrosine decarboxylase (3). Both require pyridoxal phosphate as coenzyme and act only on the L-form of the amino acid but, as known from the work of Blaschko and his colleagues (1), they have somewhat different substrate structural requirements. Dopa decarboxylase attacks ortho- or meta-hydroxyphenylalanine; tyrosine decarboxylase acts on the meta- and para-isomers, and is virtually inert towards the ortho- compound. The general applicability of these findings to the case of dopa isomers has already been verified by comparison of the action of the two enzymes on five isomers of dopa (see Table II for references).

As pointed out previously (7), 2,6-dopa is a special case in that its two ortho-phenolic groups should, by extrapolation from the results with the monohydroxylated compounds, render it susceptible to decarboxylation by dopa decarboxylase but not by the bacterial enzyme. The present report concerns the decarboxylation of this isomer, the remaining member of the dopa series. Furthermore, since only qualitative data on 2,3-dopa have been given in this respect (2) its rate of decarboxylation is also presented.

Experimental

Enzymes and Reaction Mixtures

Aqueous extracts of guinea pig kidney, prepared according to the method of Schales and Schales (6), were employed as a source of the mammalian dopa decarboxylase. The reaction mixtures have been described previously (7). Gas evolution from the substrate in the presence of the extract was measured manometrically in the Warburg apparatus at pH 6.8 and 38° C., with nitrogen

[1] *Manuscript received April 20, 1954.*
Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. The work was supported by a grant from the Dominion Provincial Mental Health Research Fund.

as the gas phase. The incubation period varied between 5 and 10 min. in various experiments. Control flasks (substrate omitted) accounted for "bound CO_2", permitting correction of the volumes of gas evolved in test flasks. Rates of decarboxylation were calculated as Q_{CO2} (microliters of CO_2 released/mgm. dry matter/hr.).

The bacterial enzyme source was an acetonized preparation of *Streptococcus faecalis R*, employed (12 mgm. per flask, supplemented with 20 μgm. of pyridoxal phosphate) as described earlier (7) at pH 5.5 and 38° C. in an air atmosphere.

Substrates

DL-3,4-Dopa was the Eastman Kodak Co. product. 2,3-Dopa and 2,6-dopa, as the racemic mixtures, were synthesized by Dr. J. P. Lambooy (5).

Results

Kidney Extract

The kidney enzyme decarboxylates the dopa isomers studied at rates decreasing in the following order: 2,3- > 3,4- > 2,6-dopa. These differences were all significant at the 5% level of probability (Table I). It will be noted that the dopa decarboxylase activity of different kidney preparations varied significantly (cf. *F* ratio for "Replicates").

TABLE I

DECARBOXYLATION OF THREE DOPA ISOMERS BY GUINEA PIG KIDNEY EXTRACTS

(Analysis of variance of data)

Source of variation	Sum of squares	D.F.	Mean square	*F* ratio
Total	1474.6185	23*	—	—
Isomers	636.0718	2	318.0359	22.47
Replicates	654.9452	8	81.8682	5.79
Remainder	183.6015	13	14.1232	(1.00)

Isomer	*n*	Mean	Standard error
2,3-dopa	7	18.67	1.41
2,6-dopa	9	7.12	1.32
3,4-dopa	8	11.29	1.24

Differences between the three pairs of means are statistically significant by Fisher's *t* test ($P = 0.05$).

** Three missing values were estimated by the method of Yates (8) to make up a 9 × 3 table for the analysis. Three degrees of freedom have therefore been subtracted from the total.*

S. faecalis R Preparation

In four experiments it was found that 2,3-dopa was decarboxylated at about one half the rate of 3,4-dopa. In the individual experiments this ratio was 50, 45, 47, and 58%. The rates were judged from the volume of gas evolved in the first 15 min. of the incubation during which period the rate was constant.

On the other hand, in numerous experiments with 2,6-dopa no gas evolution was noted even with supplements of coenzyme up to 200 μgm. pyridoxal phosphate per flask.

Discussion

Blaschko has suggested (1) that the phenolic groups in tyrosine isomers function in hydrogen-bonding of substrate to apoenzyme. Thus, a given compound in the series can unite with the apoenzyme and will be decarboxylated if it possesses an hydroxyl on the ring in an appropriate position. The results with 2,3- and 2,6-dopa fully confirm Blaschko's empirical rules of substrate specificity when these are extrapolated to the case of the dopa compounds. Particularly striking is the case of 2,6-dopa in which the prediction (7) that it is a substrate for the mammalian enzyme only has been borne out in the present work.

TABLE II

RELATIVE RATES OF DECARBOXYLATION OF DOPA ISOMERS*

Dopa isomer	Reference	Tyrosine decarboxylase	Dopa decarboxylase
2,3	2	53	165
2,4	7	113	71
2,5 = 3,6	1, 7	4	88
2,6	—	0	53
3,4	1, 7	100	100
3,5	—	18	24

* *The data shown are taken from Reference 7 and the present paper.*

Some further relationships may be deduced from Table II in which the data on all six dopa isomers are collated. Of the two *o*-, *m*-substituted compounds, 2,3- and 2,5-dopa, the former bears the groups on adjacent carbons and is considerably more rapidly decarboxylated than the latter with the groups contralaterally situated. This is observed with both types of enzyme and signifies clearly that the results with tyrosine isomers cannot be applied additively to the dopa series. The same may be said about 3,5- (= m,m-)-dopa; its rate of decarboxylation is low relative to most of the other isomers.

Thus, the pattern of substrate specificity of dopa and tyrosine decarboboxylases observed with the monohydroxyphenylalanines is qualitatively applicable to the dopa series. The factors determining the specific rate of decarboxylation, however, are not entirely clear. From the data of Table II the relative position of the two hydroxyl groups certainly plays a role. Other factors involved may be the nature of the polar groups on the apoenzyme serving to form hydrogen bonds with substrate functional groups; and the relative polarity of the dopa isomers themselves.

Acknowledgments

It is a pleasure to thank Dr. J. P. Lambooy for a generous gift of 2,3- and 2,6-dopa; Dr. W. W. Umbreit for a supply of the tyrosine decarboxylase preparation and pyridoxal phosphate; and Miss E. Townsend and Miss G. Hansen for technical assistance.

References

1. BLASCHKO, H. Biochim. et Biophys. Acta, 4 : 130. 1950.
2. BLASCHKO, H. and LANGEMANN, H. Biochem. J. (London), 48 : vii. 1951.
3. GALE, E. F. Biochem. J. (London), 34 : 846. 1940.
4. HOLTZ, P., HEISE, R., and LÜDTKE, K. Arch. exptl. Pathol. Pharmakol. 191 : 87. 1938.
5. LAMBOOY, J. P. J. Am. Chem. Soc. 76 : 133. 1954.
6. SCHALES, O. and SCHALES, S. Arch. Biochem. 24 : 83. 1949.
7. SOURKES, T. L., HENEAGE, P., and TRANO, Y. Arch. Biochem. and Biophys. 40 : 185. 1952.
8. YATES, F. Empire J. Exptl. Agr. 1 : 129. 1933.

DIRECT CURRENT POTENTIAL AND pH OF SEVERAL VARIETIES OF SKIN NEOPLASMS[1]

By W. R. Inch[2]

Abstract

A study of 36 subjects having skin neoplasms of three degrees of malignancy is described. The hydrogen ion concentration was determined using a Beckman model G pH meter with glass and calomel electrodes while d-c. potentials were measured using a d-c. vacuum tube amplifier and silver – silver chloride electrodes.

The results indicate that there is no correlation between malignancy and absolute value of potential, but a correlation between malignancy and pH difference (pH neoplasm − pH normal) and between malignancy and the 'extra positivity' (absolute d-c. potential − 0.059 pH difference) does exist.

Introduction

Langman and Burr (8, 9) in 1947 carried out measurements of the electrical potential exhibited by tumors of the cervix and concluded that nearly all (98.7% of 75) cases with cancer showed an electronegativity, while control subjects showed a definite electropositivity (81.9% of 353). Potentials were measured between the tumor and a point slightly above the symphysis pubis.

Dalgaard and Thygesen (6) in 1950 and Max *et al.* (10) in 1953 using slightly modified methods made similar determinations which showed that approximately seventy-five per cent of malignant tumors were electropositive, but this could not be attributed to their malignancy, since similar results were obtained in subjects without tumors. Their conclusion was that such a test could not be used reliably to distinguish between malignant tumors of the cervix and normal cervices.

Burrows *et al.* (4) in 1942 carrying out electrical measurements on the wound potentials of rats and Barnes (2) in 1945 making similar measurements on humans both concluded that a positive potential existed after wounding the skin and the latter showed that this potential disappeared during the healing process. Later Burrows *et al.* (5) investigated the potential differences in the skin of mice during carcinogenesis and found significant changes in the pooled results (positive potential) but not in the results from individual animals.

Following the investigation of normal skin hydrogen ion concentration by the author (7) a series of measurements on the pH and electrical activity of skin lesions was carried out. This was designed to test the correlation between these variables and the degree of malignancy as diagnosed by biopsy, and also to extend the investigation of the above authors to include cancer of the skin.

[1] *Manuscript received April 20, 1954.*

Contribution from the Department of Biophysics, Medical School, University of Western Ontario, London, Ontario. This work was supported by a research grant from the National Cancer Institute of Canada.

[2] *Holder of a Fellowship from the National Cancer Institute of Canada.*

Method

The technique which was used to determine the hydrogen ion concentration of healthy skin and which has been described in detail in a recent paper (7) was employed without modification to obtain the pH of skin neoplasms. The pH differences which are given in the observations refer to (pH neoplasm − pH normal).

The potential measurements were made using silver – silver chloride electrodes in conjunction with a d-c. amplifier designed and built in the laboratory. The former were made from No. 20 (B and S) silver wire coiled as shown in Fig. 2 and electroplated in a 0.15 *M* solution of hydrochloric acid for approximately one hour at 25 ma. These were short-circuited and stored in 0.12 *M* potassium chloride solution until ready for use. In all cases differences in the electrode potentials did not exceed 0.4 mv. and usually were of the order 0.2 mv. A circuit diagram of the amplifier is shown in Fig. 3 and a general view of the instrument in Fig. 1. This apparatus proved to be extremely stable and free from drifting (after an initial warm-up period of 5 to 10 min.), and since in the design there was a very low drain from all of the dry cells, little change in the calibration occurred during the battery life (1.40 mv./mm. to 1.28 mv./mm. in four months). Calibration of the amplifier was performed before each potential measurement by means of a 1.5 v. dry cell and an appropriate resistance circuit, shown in Fig. 3, which had previously been standardized using a Rubicon (No. 2700) precision type potentiometer. With this instrument the potentials could be read directly from the galvanometer with an accuracy of ± 0.25 mv. after a period of three to five seconds required for equilibrium to be reached. This factor plus the constant input impedance of the amplifier were considered the main advantages which it had over a potentiometer by itself.

In most determinations one electrode was placed on the lesion and the other on the symmetrically opposite part of the body. In the remainder of the measurements the electrodes were rigidly held at a distance of 5 cm., one on normal and the other on abnormal tissue. The normal gradient measured on the opposite side of the body was used to correct the reading but in no case did this exceed 2.0 mv. in magnitude. In all determinations the electrodes were reversed and the data given are the averages of the two corrected readings which differed by less than a millivolt. This technique assured the elimination of the electrode potential, which could otherwise affect readings of small magnitude.

The resultant 'extra-positivity' of the lesion, given in the observations, is the absolute potential minus the potential contributed by the hydrogen ion concentration difference i.e. as given by the reduced form of the Nerst equation:

$$E_1 - E_2 = 0.059\ (pH_1 - pH_2)$$

where $E_1 - E_2$ represents the difference in potential between points 1 and 2 of skin having a hydrogen ion concentration of pH_1 and pH_2 respectively.

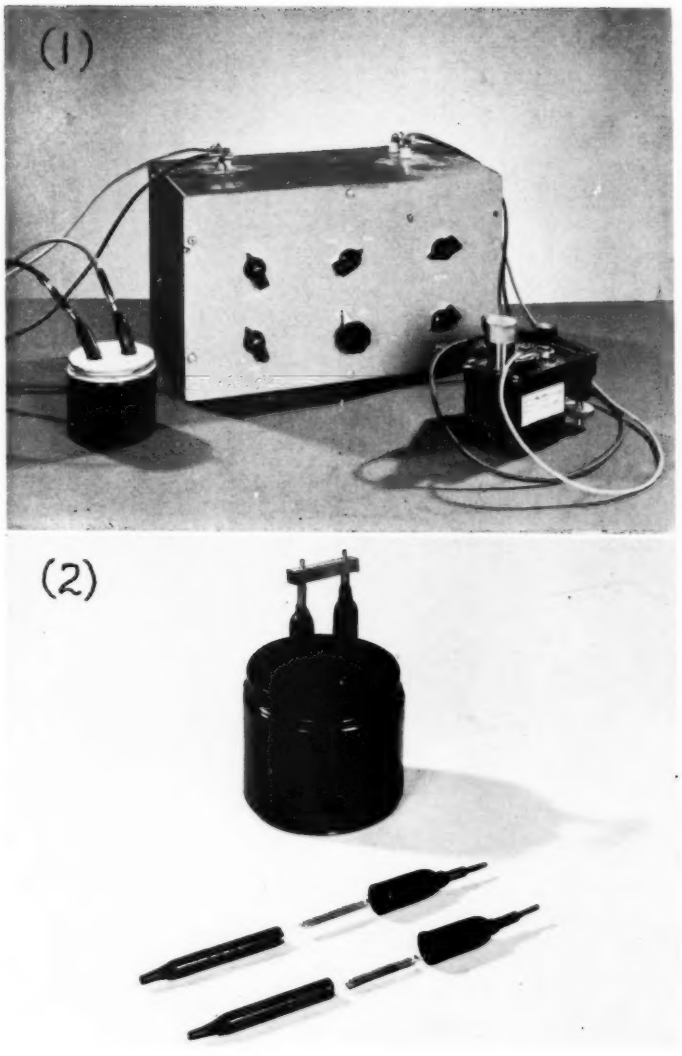

FIG. 1. D-c. amplifier used for potential measurements.

FIG. 2. Silver – silver chloride electrodes:—above—shorted in 0.9% potassium chloride.
—below—prior to plating.

A group of 36 subjects (20 males and 16 females) obtained from the local Cancer Clinic was used in the study. These patients were selected at random from the clinic after preliminary diagnosis had been made but prior to any form of therapy. Those lesions which were badly ulcerated or which had been treated by topical application of drugs, etc. were excluded from the study. Subjects were placed in a supine position and their skin was washed with ethyl ether and then moistened with a contacting solution of 0.12 molar potassium chloride unbuffered but at a pH of 6.7 (3, 7). The electrodes were placed directly in contact with these moistened areas and held in place by insulating handles and clamps.

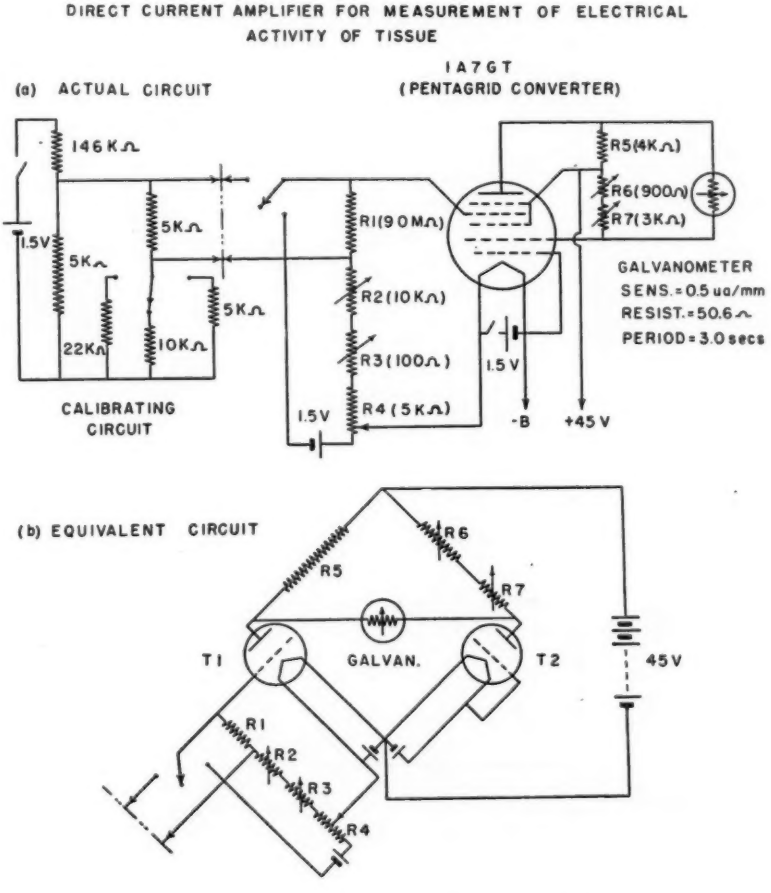

FIG. 3. Schematic circuit of the d-c. amplifier.

TABLE I

SUMMARIZED DATA

Group	Number of cases (*n*)	D-c. potential (mv.)			pH difference from normal (pH units)			'Extra positivity' D-c. potential − pH difference (mv.)		
		Av.	S.D.	S.E.M.	Av.	S.D.	S.E.M.	Av.	S.D.	S.E.M.
0	14	+ 8.70	± 5.4	± 1.4	− 0.091	± 0.350	± 0.094	+ 2.75	± 19.5	± 5.2
I	19	+ 9.66	± 8.9	± 2.0	+ 0.145	± 0.351	± 0.080	+ 18.61	± 22.2	± 5.1
II	3	+ 1.86	± 12.5	± 8.8	+ 0.803	± 0.551	± 0.390	+ 49.40	± 18.8	± 13.3
Total	36	+ 8.64	± 11.9	± 2.0	+ 0.108	± 0.031	± 0.005	+ 15.10	± 24.5	± 4.1

Av. = *Arithmetic mean.*
S.D. = *Standard deviation of arithmetic mean.*
S.E.M. = *Standard error of arithmetic mean.*

Observations

The data obtained are summarized in Table I. Distribution bar charts for absolute potential difference, absolute pH difference, and the 'extra positivity' are given in Fig. 4 (*a*), (*b*), and (*c*) respectively. These are subdivided into three groups according to the degree of malignancy. Group 0 comprises those tumors which show few or no signs of malignancy and includes such types as senile keratosis, papillomata, and several basal cell carcinomata of very slight malignancy. Group I comprises those tumors which were diagnosed by biopsy as grade I malignancy and includes such types as squamous and basal cell carcinoma. Group II comprises those tumors which were diagnosed by biopsy as grade II malignancy. The cases studied were all squamous cell carcinomata.

The range of the potentials exhibited by the neoplasms was -8.3 to $+26.6$ mv. with 31 out of 36 (86%) showing a positivity. However, no significant correlation between this absolute potential and the degree of malignancy could be demonstrated.

The hydrogen ion concentration of the normal skin varied from 4.9 to 6.2 pH units in good agreement with the results of other workers (3) while the range for the skin tumors was 5.4 to 7.7 pH units. In 22 out of 36 patients the tumor was more basic, being found predominantly (17 out of 22) in the more malignant groups I and II. Statistical analysis of the results indicates

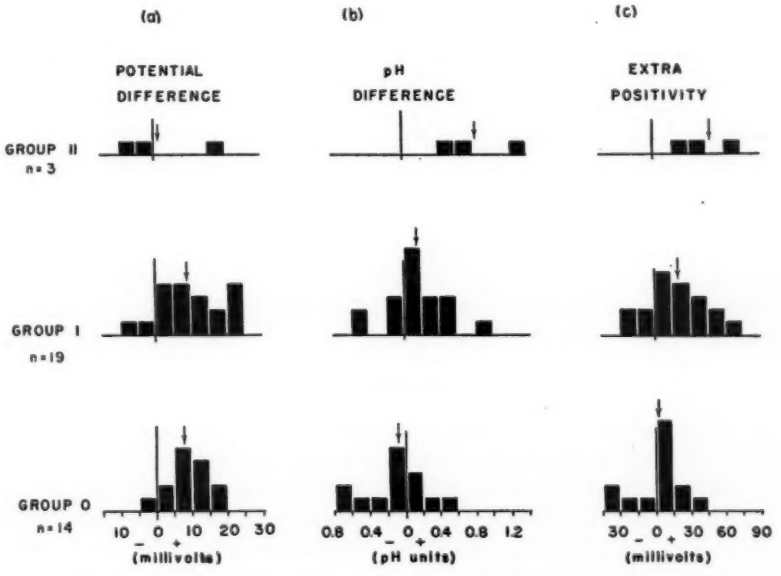

FIG. 4. Distribution bar charts of the data; arrows indicate the arithmetic mean.

that there is a significant correlation ($r = + 0.58 \pm 0.11$) between the pH differences of the tumor (pH tumor − pH normal) and the degree of malignancy.

The 'extra positivity' of the tumor calculated as shown under the method was considered to be a better indication of the actual tumor potential than that measured by silver – silver chloride electrodes alone, since the hydrogen ion activity of the tissue contributes greatly to its potential level. Statistical analysis of the data showed that there was a fair correlation ($r = + 0.49 \pm 0.13$) between 'extra positivity' and malignancy.

Discussion

The group studied was too small to allow definite conclusions to be drawn but at the present time one can say that the measurement of the absolute d-c. potential alone cannot be used to diagnose reliably the malignancy of a skin tumor. It may be that this potential is analogous to the injury potential described by Barnes and others (1, 11), since although it is much smaller in magnitude it decreases in a similar manner during the healing process. Daily measurements were observed in only two of the above cases and hence the data were not included here. It is noteworthy however that the rate of potential decrease expressed as a percentage (the healing index as defined by Barnes) was approximately two per cent per day in one case and four per cent per day in the other.

Skin is normally more acid than serum (3, 7), therefore the decrease in the hydrogen ion concentration of the neoplasms with their increased malignancy could be due to their increased vascularity and excess tissue fluid. The fact that the pH in some cases was even more basic than blood can be explained on the grounds that basic products of tissue degeneration are not cleared rapidly enough and hence become concentrated in the tumor. It should be pointed out that although patients showing visual signs of tissue necrosis were excluded from the study, the several which were measured showed very definite basic reactions, having a tumor pH greater than 7.0. Whether the tendency toward basicity potentiates necrosis or whether it results from necrotic detritis and bacterial action beneath the stratum corneum cannot be established from the above data, but this aspect of the problem should be investigated in a further study.

Conclusion

1. The absolute d-c. potential or skin tumors cannot be correlated quantitatively with malignancy although skin neoplasms in general exhibit a positive potential (31 out of 36) with respect to normal skin.

2. The difference between the hydrogen ion concentration of neoplasms and normal skin shows a significant correlation with malignancy, tumors being more basic than normal tissue. The coefficient of correlation is $r = + 0.58 \pm 0.11$.

3. The 'extra positivity' of the tumor defined as the absolute potential minus the calculated potential due to pH differences, shows a significant correlation with malignancy. The coefficient of correlation $r = +0.49 \pm 0.13$.

Acknowledgments

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References

1. Arey, L. B. Physiol. Rev. 16 : 327. 1936.
2. Barnes, T. C. Am. J. Surg. 69 : 82. 1945.
3. Blank, I. H. J. Invest. Dermatol. 2 : 67; 75; 231; and 235. 1939.
4. Burrows, H., Iball, J., and Roe, E. M. F. Brit. J. Exptl. Pathol. 23 : 253. 1942.
5. Burrows, H , Roe, E. M. F., and Schober, B. Cancer Research, 5 : 524. 1945.
6. Dalgaard, S. and Thygesen, J. E. Acta Radiol. 34 : 488. 1950.
7. Inch, W. R. and Burton, A. C. Can. J. Med. Sci. 31 : 127. 1953.
8. Langman, L. and Burr, H. S. Science 105 : 209. 1947.
9. Langman, L. and Burr, H. S. Am. J. Obstet. Gynecol. 57 : 274. 1949.
10. Max, L. W., Mauss, E. A., Day, E., and Rhoads, C. P. Cancer, 6 : 77. 1953.
11. Snodgrass, J. M. and Davis, H. Am. J. Physiol. 129 : 468. 1940.

ULTRAVIOLET LIGHT ABSORPTION IN NORMAL AND IN PATHOLOGICAL CEREBROSPINAL FLUIDS[1]

By Ernest Kovacs

Abstract

Ultraviolet spectrophotometric studies were described in 402 cerebrospinal fluids (CSF's). Normal specimens exhibited a hyperbolic curve with end-absorption but no selective absorption. In CSF's of neurologically normal hospital patients absorption maxima were found, possibly dependent upon moderate increase of proteins (PR). In centrifuged meningitis samples peaks were demonstrated, scattered over the spectral region investigated. Fifteen per cent of the CSF's in this group exhibited absorption curves similar to the normal. In contrast, all but 2% of the poliomyelitis specimens exhibited absorption bands. The distribution of the maxima was compact; none under 2500 Å., and the vast majority in the middle zones from 2600 to 2900 Å., caused in part probably by nucleic acid (NA) and PR, or their derivatives. The samples of neurosyphilis, miscellaneous neurological, and psychotic cases exhibited a variety of absorption bands. In degenerative processes also selective bands suggesting some steroid compounds appeared. Some special features of ultraviolet spectrophotometry of the CSF were discussed. The described technique supplemented by chemical methods and applied critically may be a useful tool in clinical laboratory work.

Introduction

Absorption spectrophotometry is useful in the demonstration of minute quantities of chemical materials. Scheid (29) and others (28) applied this technique to the examination of the CSF. Spiegel-Adolph and Wycis (33) claimed the presence of NA in CSF's in which routine clinical laboratory tests gave negative results. These findings are not generally accepted (34). Owing to the biological importance of these substances, we believe that every effort should be made to elucidate the behavior of Na in such an accessible part of the central nervous system as the CSF. The main interest in this study was the detection of NA in various pathological conditions, especially in poliomyelitis.

Methods

The preliminary assays were made with a Hilger Uvispeck[2] spectrophotometer. A Beckman D. U. quartz spectrophotometer was used for all the absorption measurements reported in this paper.

Fresh or deep-frozen samples were investigated. CSF (0.5 ml.) was diluted to 4 ml. with physiological saline in quartz cells of 1 cm. light path. A small number of specimens were used undiluted; a few samples which had high protein concentration or in which CSF was scarce were diluted 16 to 80 times. The deep-freezing did not affect the optical properties of the samples.

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[2] *Courtesy of Dr. R. C. Parker and Mr. G. M. Healy.*

Centrifuged CSF's were used in the great majority of the assays. Readings were made against a physiological saline blank, beginning at 2200 Å. and changing the wave length scale by 100 Å. up to 3000 Å. In the middle zone we determined additional values at 2550, 2650, and 2750 Å. In each clinical group detailed measurements were made on a few specimens from 2100 Å. to 4000 Å. The absorption maxima were recorded on curves. The effect of hydrogen-ion concentration on the ultraviolet light absorption of CSF was also investigated. In some assays borate and other buffers were used in place of physiological saline to ensure the desired pH values. The effect of spontaneous pH changes was studied simultaneously in incubated, diluted, buffered, and unbuffered CSF's. Determinations were made before and after incubation and the curves compared.

As a check, tests for NA were also made by colorimetric methods. Dische's carbazole (7); Bial's orcinol reagent (25) for pentose nucleic acid (PNA); and Dische's diphenylamine (7) and Feulgen's leucofuchsin reagent (35) for desoxyribonucleic acid (DNA) were employed.

The majority of specimens were derived from infants and children: for example, the meningitis specimens with only one exception were from the young age groups. The polio and virus samples were mostly from children, but included some from adults. The normal and neurologically normal groups were mixed, infants and children predominating. The syphilis group was composed of adults and the great majority of the CSF's of neurological and psychotic cases came from adults. Diagnoses and clinical laboratory findings of the hospital staff were accepted in these groupings. Protein, sugar, and cell count determinations were all made in the clinical laboratories.

Results

The 402 CSF's examined fell into six clinical groups: normal and neurologically normal; meningitis; poliomyelitis and other virus diseases; syphilis; miscellaneous neurological conditions; psychoneuroses.

The synopsis of the results is shown in Table I. The number and distribution of all the specimens and of all the absorption bands is presented. The absorption bands found at the various wave lengths constitute the right side of the table. The sum of multiple maxima given by individual samples is shown in brackets at specified wave lengths. Illustrative examples of the results in the various clinical groups are shown in Table II and Fig. 1. Fig. 2 illustrates the effect of incubation on the ultraviolet light absorption of the CSF and Fig. 3 presents a few results of assays with known amounts of added substances.

Normal CSF did not exhibit any selective absorption, as found in 17 specimens of the first group (Table I, Table II, and Fig. 1). The absorption curve is hyperbolic (Fig. 1) without any definite peak. CSF specimens of non-neurological conditions on the other hand show absorption maxima, in most instances at 2800 Å., probably due to the presence of PR (6, 32). Extinction was demonstrated exceptionally at other wave lengths: one sample

TABLE I

SYNOPSIS OF THE RESULTS OF ULTRAVIOLET LIGHT ABSORPTION IN 402 CSF's

Group	Total No. of samples	No. of samples with			No. and distribution of maxima at wave length in Å.														
		No selective absorption	One absorption maximum	Two or more absorption maxima	2100	2200	2300	2400	2450	2500	2550	2600	2650	2700	2750	2800	2900	3000	Total of maxima
1. Normals and neurologically normals	50	17	28	5	0	0	0	0	0	1 (1)*	0	0	1 (1)	4 (3)	0	31 (4)	1 (1)	0	38 (10)
2. Meningitis	100	15	60	25	0	1	0	2 (2)	1	2 (2)	3 (2)	17 (10)	19 (10)	20 (6)	2	39 (14)	5 (5)	1 (1)	112 (52)
3. (*a*) Poliomyelitis	120	2	84	34	0	0	0	0	0	4 (4)	3 (3)	16 (8)	25 (15)	18 (16)	2 (1)	76 (21)	12 (4)	2 (2)	158 (74)
(*b*) Viruses other than polio	14	0	9	5	0	0	0	0	0	0	1 (1)	2	4 (2)	6 (3)	0	4 (3)	3 (2)	0	20 (11)
4. Syphilis	50	5	22	23	0	0	0	1 (1)	0	2 (1)	3 (3)	6 (4)	9 (5)	10 (5)	3 (1)	32 (25)	3 (2)	0	69 (47)
5. Miscellaneous neurological samples	50	3	22	25	1 (1)	0	0	2 (2)	0	6 (5)	0	7 (5)	19 (11)	9 (8)	4 (2)	18 (11)	10 (9)	2 (2)	78 (56)
6. Psychoses	18	2	11	5	0	0	0	0	0	1 (1)	0	1 (1)	4 (3)	6 (2)	2 (1)	6 (3)	2 (2)	0	24 (13)

* *The figures in brackets indicate the number of multiple maxima.*

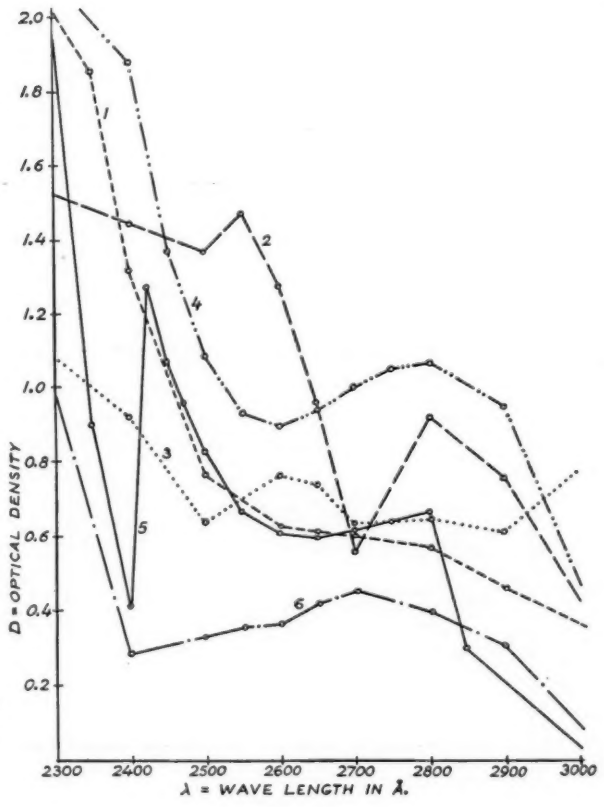

FIG. 1. Illustrative curves from the six clinical groups.

1 – – – – – Normal CSF
2 —— —— Meningitis
3 ····· Poliomyelitis
4 —— ·· —— Syphilis
5 —————— Brain abscess
6 —— · —— "Hysteria"

only had a maximum at 2650 Å. (26). Color reactions for NA gave negative results in this group. The CSF specimens of Group 1 were from 17 normal persons and 33 neurologically normal. The latter were from nasopharyngitis, tonsillitis, pneumonia, gastroenteritis, adenitis, infectious mononucleosis, typhoid, influenza, and cured syphilis cases.

Table II and Fig. 1 illustrate characteristic absorption curves of meningitis specimens. Fifteen samples did not show any absorption band in this group (Table I). The majority of specimens exhibited selective absorption between 2700 and 3000 Å., possibly because of increased PR and/or amino acids (6, 14, 15, 32). The large number of maxima between 2550 and 2700 Å. is noteworthy, suggesting the presence of NA derivatives and/or of other absorbing material (34). Another interesting feature is an occasional selective

TABLE II

ULTRAVIOLET LIGHT ABSORPTION IN THE CSF

(Illustrative examples)

Group	Diagnosis	Laboratory notes	Wave length maxima Å.	Maxima of optical density D
1	Normal fluid	Neg. findings; diluted 1 : 8	0	0
	Tracheitis	4 cells; diluted 1 : 8	0	0
	Lobar pneumonia	Neg. findings; diluted 1 : 8	2650; 2900	0.060: 0.078
	Typhoid	43 cells; diluted 1 : 8	2800	0.110
2	Meningococcal meningitis	High cell count; Pá pos.; diluted 1 : 8	2600; 2700; 2900	0.092; 0.077; 0.078
	Purulent staphylococcal meningitis	High cell count; Pá pos.; undiluted	2550; 2800	0.147; 0.925
	Pneumococcal meningitis	High cell count; Pá pos.; undiluted	2650	1.112
	Influenzal meningitis	High cell count; Pá pos.; diluted 1 : 8	2700	0.094
	E. coli meningitis	High cell count; Pá pos.; diluted 1 : 8	2600	2.00
3	Nonparalytic poliomyelitis	No cells; Pá neg.; diluted 1 : 8	2650; 2800	0.102; 0.164
	Paralytic poliomyelitis	1000 cells; Pá pos.; diluted 1 : 8	2600; 2700; 2800	0.058; 0.068; 0.068
	Bulbar poliomyelitis	18 cells; Pá neg.; diluted 1 : 8	2900	0.108
	Polioencephalitis	5 cells; Pá neg.; diluted 1 : 8	2600; 2800	0.100; 0.014
	Mumps	42 cells; Pá neg.; diluted 1 : 8	2650; 2800	0.095; 0.061
	Measles encephalitis	20 cells; Pá neg.; diluted 1 : 8	2640; 2750	0.145; 0.152
	Lymphocytic meningitis	62 cells; Pá neg.; diluted 1 : 8	2650	0.060
4	Primary syphilis	Neg. findings; diluted 1 : 8	2650; 2800	0.113; 0.104
	Secondary syphilis	Neg. findings; diluted 1 : 8	2750	0.290
	Neurosyphilis	CSF Wass. R. pos.; diluted 1 : 8	2650; 2800	0.204; 0.218

TABLE II—(*Concluded*)

ULTRAVIOLET LIGHT ABSORPTION IN THE CSF—*Concluded*

(Illustrative examples)—*Concluded*

Group	Diagnosis	Laboratory notes	Wave length maxima Å.	Maxima of optical density D
5	Genuine epilepsy	Neg. findings; undiluted	2650	0.447
	Head injury	Neg. findings; undiluted	2600; 2900	0.525; 0.499
	Neurofibromatosis (Recklinghausen's disease)	No cells; prot. 16 mgm. %; undiluted	2650; 2700	0.0985; 1.985
	Brain tumor (unverified)	No cells; prot. 52 mgm. %; diluted 1 : 8	2500; 2800; 2900	0.035; 0.037; 0.029
	Brain atrophy	X-ray and EEG pos.; diluted 1 : 8	2500; 2650; 3000	0.020; 0.032; 0.011
	Lat. sinus thrombosis	198 cells; Pá pos.; diluted 1 : 8	2650; 3000	0.020; 0.018
	Cerebral abscess	20 cells; prot. 277 mgm. %; diluted 1 : 20	2650; 2900	0.080; 0.046
	Hydrocephalus	No cells; prot. 522 mgm. %; diluted 1 : 2	2700; 2900	0.150; 0.959
6	Anxiety neurosis	Neg. findings; undiluted	0	0
	Schizophrenia catatonic	Neg. findings; diluted 1 : 8	2650; 2800	0.065; 0.055
	Schizophrenia	Neg. findings; diluted 1 : 8	2500; 2700	0.080; 0.078
	Paraphrenia	No cells; prot. 17 mgm. %; undiluted	0	0
	Hystero-epilepsy with personality change	Neg. findings; undiluted	2650; 2800	1.280; 1.500
	Posttraumatic psychosis	No cells; prot. 36 mgm. %; undiluted	2700	0.726
	Anxiety state with brain atrophy	X-ray pos.; diluted 1 : 8	2600; 2700; 3000	0.015; 0.016; 0.023

absorption in the lower spectral region studied (2200–2400 Å.), implying the presence of steroid compounds (26). The etiological subgroups comprised: 33 meningococcal, 19 purulent (strepto- and staphylococcal), 20 pneumococcal, 20 influenzal, and 8 *E. coli* meningitis. In one *E. coli* meningitis specimen with 80,000 leucocytes per cubic millimeter was found 800 mgm. % DNA and 400 mgm. % PNA with the diphenylamine and with the Bial reagent respectively.

Table II and Fig. 1 present some characteristic examples of the various poliomyelitis specimens. In this group 2 specimens only of 120 did not show absorption bands (Table I) and none were found in the lower regions under 2500 Å. In the middle zone the number of maxima was similar to the meningitis group, but in the higher regions (2800–2900–3000 Å.) a larger number of peaks was found. The poliomyelitis samples fell into the following groups: 26 nonparalytic, including suspects, 21 paralytic, 21 bulbar, 2 polioencephalitis. Color reaction for NA occasionally gave positive results. The small number of specimens from other virus diseases in this study exhibit characteristics similar to those of the poliomyelitis group.

In syphilis the number of specimens without selective absorption is relatively low. The remaining samples show 1 maximum in 22 and 2 or more maxima in 23 (Table I). The neurosyphilis specimens exhibited more numerous absorption bands than those of early syphilis. The absorption bands are scattered in the various spectral regions studied (Fig. 1, Table I, Table II), indicating the presence of a variety of substances.

The miscellaneous neurological group is a conglomerate of diagnoses, the majority made up as follows: 10 epilepsy, 14 brain tumor, 6 hydrocephalus, 5 head injury, 3 brain atrophy. NA was demonstrated in most of the tumor (5) specimens and in many of the epilepsy and posttraumatic samples. The 4 brain atrophy specimens exhibited various absorption maxima. It is noteworthy that a high proportion of CSF's exhibited maxima at 2900 or 3000 Å. (Table I). In a brain abscess patient a sharp peak was observed in the CSF at 2420 Å. which may be due to some steroid compound; the lower maximum at 2800 Å. is due to PR. The great majority of psychotic (Table II, Fig. 1) specimens showed selective absorption, especially in the case of organic psychoses.

Effect of Incubation

All the above results of absorption measurement were carried out on fresh or on frozen specimens. Long standing at room temperature, and incubation at various temperatures and periods of time, modify the shape of the absorption curves (Fig. 2). One example on this figure, a poliomyelitis specimen, illustrates the complete disappearance, after incubation, of the absorption bands originally present. An epilepsy specimen under similar circumstances exhibited a shift of the absorption maximum from 2700 to 2900 Å., with the simultaneous decrease of the optical density. The third example from an "anxiety state" exhibited considerable flattening of the absorption curve and

illustrates, along with the other samples, marked changes in the substances responsible for selective absorption of the CSF. Higher temperatures had similar but intensified effect. The pH of the routine mixture of CSF and saline was around 8 and all the readings were made in this slightly alkaline milieu. Extreme pH ranges fixed by buffers may change the shape of absorption curves. We are continuing these assays and intend to publish the findings in a separate paper. The spontaneous pH changes of the CSF were also investigated. An increasing alkalinity was observed on standing or on incubation ranging from pH 7.3 to 9.5 in an extreme case. This finding is in agreement with that of others (19) and is explained by the loss of carbon dioxide. The change of pH may influence the autolysis and/or spontaneous hydrolysis in the CSF, illustrated in Fig. 2. The results of these illustrative experiments showing the effect of pH and of room and incubation temperatures emphasize the importance of using fresh or fresh frozen specimens for ultraviolet spectrophotometry.

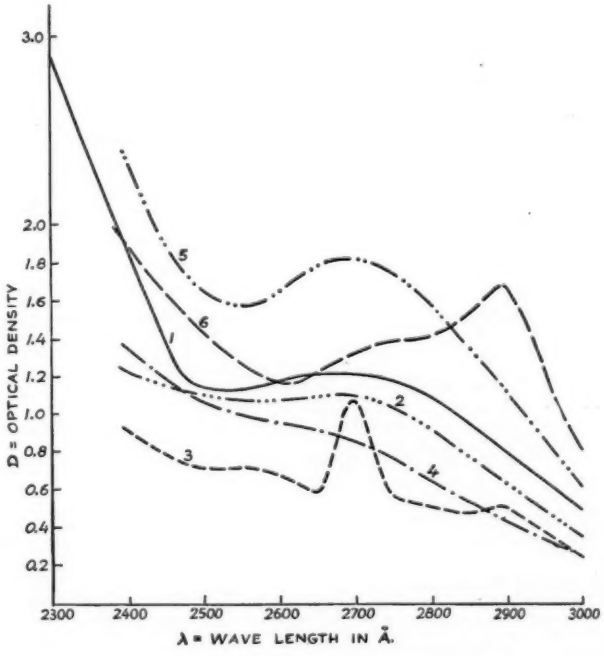

FIG. 2. Spontaneous hydrolysis or autolysis (at 37° C.)

1 ———— Anxiety state, zero-reading
2 — · · · — Anxiety state, 96 hr. incubation
3 — — — — Paralytic polio, zero-reading
4 —— · —— Paralytic polio, 96 hr. incubation
5 —— · · —— Epilepsy, zero-reading
6 —— — —— Epilepsy, 96 hr. incubation

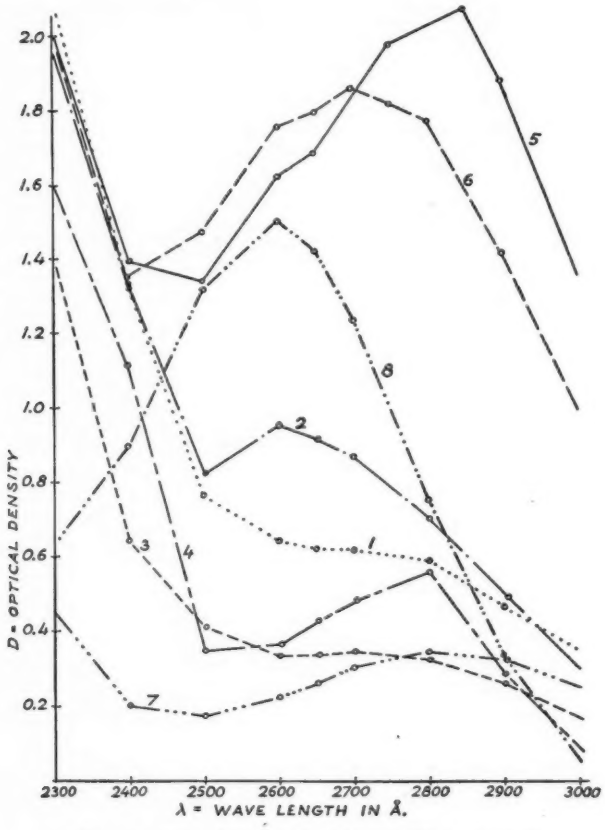

FIG. 3. Detection of added NA and PR in the CSF.

1 ····· Normal CSF
2 —— · —— NA mixture added, in 0.0025% final concentration (PNA and DNA)
3 — — — — Normal CSF
4 —— - —— Gamma-globulin added, in 0.0125% final conc.
5 ———— CSF, undiluted, containing large amount of PR
6 —— —— NA added (PNA and DNA), in 0.005% final conc.
7 —— ··· —— 0.5 ml. of the same CSF, in phys. saline (diluted 8 times)
8 —— ·· —— 0.5 ml. CSF and NA (PNA and DNA), in 0.0075% final conc.

Detection of Added Substances

In another set of experiments we simulated the spectrophotometric behavior of many of the pathological CSF's by the addition of both PNA[3] and DNA[4] to normal CSF specimens, and similarly, after the addition of purified serum albumin or gamma globulin[5]. Fig. 3 presents illustrative examples of our results. Added NA and human serum proteins can be detected in the CSF,

[3] *PNA was purified by us from Merck's commercial brand.*
[4] *Highly polymer DNA kindly supplied by Dr. G. C. Butler.*
[5] *Kindly supplied by Mr. M. Poulik.*

giving characteristic extinction at 2600 Å. for NA and at 2800 Å. for PR respectively. The shape of the curves is similar to that seen in various pathological specimens. The normal samples before the addition of substance gave no selective absorption, as can be seen in the illustrations. In the case of CSF exhibiting intense maxima at 2800 Å., both in undiluted and in diluted state, a very small amount (0.005%) of NA mixture was added which shifted the peak to 2700 Å. When the NA concentration was increased to 0.0075%, the maximum was found at 2600 Å. (Fig. 3). Similar observations were made in normal CSF's after addition of very small amounts of NA and PR, separately or mixed.

During the investigation of standard NA solutions, it was found, in agreement with others (4, 10), that the PNA gave higher optical density at 2600 Å. than did DNA. The nature of a solvent is another factor which has to be considered: NA solutions in water exhibited higher peaks than in buffers. The degree of polymerization caused differences also in the extinction value of NA: commercial preparations gave higher peaks than the highly polymerized. Depolymerization by nuclease (23) caused elevation of the absorption maximum. The graphical illustration of these experiments is omitted to save space.

Discussion

We were chiefly concerned in this study with the detection of NA. Sugars, polysaccharides, aldehydes, etc. interfere with the color reactions for NA in the body fluids (27). The carbazol test, however, as used by Seibert and Atno (31) followed by spectrophotometry of the color developed was applied with success to differentiate between carbohydrates and NA in serum. We tried this and similar techniques for the determination of polysaccharides in the CSF. The direct light absorption measurement however is more simple, carbohydrates do not interfere, and a small amount of CSF is required. The disadvantages of this technique are that its specificity is not great: it has to be supplemented by chemical analysis to distinguish between various substances giving selective absorption in this region.

The absorption curve of normal CSF is similar to very diluted PR solutions (10). The end-absorption (29, 34) found above 2300 Å. may be due to these substances in a concentration too low to give selective absorption. In non-neurological diseases such as acute tonsillitis or nasopharyngitis the spectrophotometer demonstrates a definite peak at 2800 Å. (Fig. 1). This suggests that in a Pândy-negative CSF there is some increase in PR even during a simple febrile condition. The absorption band at 2600 Å., on the other hand, does not appear in the CSF until there is a more serious disease of the meninges or of the central nervous system. The extinction of the chromophoric group of NA and some derivatives is so strong at 2600 Å. that minute quantities give a definite maximum (10, 2, 4). In a series of our own assays, 0.00002% of RNA in water was detectible by ultraviolet spectrophotometry. This may mean that as a rule the concentration of NA may be below this level or lacking completely in normal and neurologically normal specimens.

The wave length maxima of PNA and DNA are identical, 2600 Å., but the density value is much higher in the former (10). Thus it is difficult to differentiate between the two types by absorption measurements only, and the peaks found by us in many CSF's may be caused by one type, or by the mixture of the two types of NA. Our assays with NA added to normal CSF simulate closely the absorption curves found in pathological CSF's showing a peak at 2600 Å. We assumed that the specimens exhibiting this band contained NA or derivatives of NA. This finding was confirmed by the fact that many specimens gave various degrees of color reactions. The majority, however, had a maximum at 2650 Å. Previous workers reported similar findings (33) and this was tentatively explained, on the basis of Lavin's (22) work, by the presence of nucleoproteins (NPR). Similarly on the addition of small quantities of NA and PR to normal CSF's, we observed a single maximum at 2700 Å. in place of two absorption bands, as might be expected, since the two substances disguise one another and shift the maximum. The conclusions of Spiegel-Adolph (33) and others were criticized by Strait *et al.* (34), who assume that all the peaks at 2650 Å. in the CSF are caused by vitamin C. This generalization however has yet to be definitely proved.

At 2500 Å., NA derivatives such as guanylic acid or guanosine (26, 13) give a definite peak. End-absorption in this region may be due to aliphatic amino acids, free or bound to PR (14, 26). The few peaks at 2750 Å. may be caused by both NA derivatives (4, 36) or by PR derivatives (32). NA, nucleotides, NPR, and derivatives give absorption bands between 2500 and 2750 Å. (2, 10, 4, 13, 26, 36). Of the substances giving maxima in this range the following are the most important to be excluded: barbital, barbituric acid (12), antibiotics (8), vitamins (26, 34), and uric acid (13).

In inflammatory conditions the high permeability of the meninges (18) may account for the appearance of NA in the spinal fluid. This may originate from disintegrated leucocytes, bacteria (1, 3), or occasionally from tissue elements (30). The high autolysis in these specimens (20) may account for the 15% exhibiting no selective absorption. It is very probable that these changes occurred *in vivo*. On the other hand the peaks corresponding to NA in polio and virus specimens have frequently been found associated with low cell count. Whether or not this latter finding is a consequence of cytolysis (24) is not established. The possibility of the presence of virus NPR and virus breakdown products in these CSF's would be an attractive hypothesis. The increased NA metabolism in polio cases evidenced by intense RNA-ase activity in the CSF, previously described by us (21), and by the presence of NA suggested in these CSF's, is noteworthy and significant (Table I). The neuronal changes (16) may give rise to one part of the ultraviolet light absorbing materials (9) in the CSF. Comparing the absorption bands between 2500 and 2750 Å., 68 maxima (43%) were demonstrated in poliomyelitis specimens and 53 maxima (47%) in meningitis specimens (Table I). The difference is not significant between the two groups in this respect. Of more importance

is the larger number (98%) of specimens without absorption bands in poliomyelitis, in contrast with the smaller number (85%) in the meningitis samples. This is in agreement with the previously observed inhibition or diminution of autolysis in CSF of polio patients (20). On the other hand, the lack of absorption band may be explained by spontaneous hydrolysis, or by erroneous diagnosis which included normal CSF's in the subgroup of nonparalytic poliomyelitis.

Generally speaking, the diminution or complete disappearance of selective absorption on standing or on incubation may be due to two factors: namely autolysis, brought about by enzymes (20) and spontaneous hydrolysis. The latter may be caused by pH changes in the CSF and by the fact that very dilute NA solutions are unstable in an alkaline milieu (11). The importance of these processes upon the "lability" of the various ultraviolet light absorbing materials (34) cannot be overemphasized. The few observations in the various subgroups of psychosis specimens are provocative and require further research. In all the CSF's of organic psychoses various absorption bands were present. The NA found in some hyperkinetic states such as hysteria, "hystero-epilepsy", and catatonic type of schizophrenia may be explained by the findings of Hyden (17) who claims that the motoneurons give up one part of their NA and PR content upon overstimulation.

Most of the results of the present investigation could be evaluated quantitatively, as seen from the actual density readings (Table II or Fig. 1), if we compare these with the extinction values of standard NA, NPR, or PR solutions. We are continuing our work on poliomyelitis specimens to correlate these findings with the clinical data.

Acknowledgments

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References

1. Belozersky, A. N. Cold Spring Harbor Symposia Quant. Biol. 12 : 1. 1947.
2. Caspersson, T. Fortschr. Zool. 2 : 276. 1933.
3. Chargaff, E. Cold Spring Harbor Symposia Quant. Biol. 12 : 28. 1947.
4. Chargaff, E., Magasanik, B., Vischer, E., Green, C., Doniger, R., and Elson, D. J. Biol. Chem. 186 : 37. 1950.
5. Cumings, J. N. Brain, 66 : 316. 1943.
6. Dhéré, C. Thèse de Paris. Fribourg. 1909.
7. Dische, Z. Mikrochemie, 8 : 4. 1930.
8. Donovick, R., Rake, G., and Fried, J. J. Biol. Chem. 164 : 173. 1953.
9. Gersh, J. and Bodian, D. J. Cellular Comp. Physiol. 21 : 253. 1943.
10. Greenstein, J. P. Advances in Protein Chem. 1 : 209. 1944.
11. Gulland, J. M. J. Chem. Soc. p. 1722. 1938.
12. Heyroth, F. F. and Loofbourow, J. R. J. Am. Chem. Soc. 56 : 1728. 1934.
13. Holiday, E. R. Biochem. J. (London), 24 : 619. 1930.

14. HOLIDAY, E. R. Biochem. J. (London), 30 : 1795. 1936.
15. HOLIDAY, E. R. and OGSTON, A. G. Biochem. J. (London), 32 : 1166. 1938.
16. HOWE, H. A. and BODIAN, D. Neural mechanism in poliomyelitis. Commonwealth Fund, New York. 1942.
17. HYDEN, H. Cold Spring Harbor Symposia Quant. Biol. 12 : 105, 154. 1947.
18. KAPLAN, I., COHN, D. F., LEVINSON, A., and HERN, B. J. Lab. Clin. Med. 25 : 495. 1945.
19. KATZENELBOGEN, S. The cerebrospinal fluid and its relation to the blood. Johns Hopkins Press, Baltimore. 1935. p. 221.
20. KOVACS, E. Can. J. Med. Sci. 31 : 358. 1953.
21. KOVACS, E. Can. J. Med. Sci. 31 : 437. 1953.
22. LAVIN, G. I., LORING, H. S., and STANLEY, W. M. J. Biol. Chem. 130 : 259. 1939.
23. LITTLE, J. A. and BUTLER, G. C. J. Biol. Chem. 188 : 695. 1951.
24. LYON, G. M. Am. J. Diseases Children, 36 : 40. 1928.
25. MEJBAUM, W. Z. physik Chem. 258 : 117. 1939.
26. MORTON, R. A. The application of absorption spectra. Adam Hilger, Ltd., London. 1942.
27. PIRIE, N. W. Brit. J. Exptl. Pathol. 17 : 269. 1936.
28. PIROSKY, J. Rev. Especialidades, 6 : 1250. 1903.
29. SCHEID, K. F. Z. ges. Neurol. Psychiat. 158 : 101. 1937.
30. SCHEINKER, I. M. J. Neuropathol. Exptl. Neurol. 4 : 164. 1945.
31. SEIBERT, F. B. and ATNO, J. J. Biol. Chem. 163 : 511. 1946.
32. SPIEGEL-ADOLPH, M. and KRUMPEL, O. Biochem. Z. 208 : 45. 1929.
33. SPIEGEL-ADOLPH, M. and WYCIS, H. T. J. Phys. Chem. 50 : 447. 1946.
34. STRAIT, J. A., AIRD, R. B., and HRENOFF, M. K. Science, 106 : 64. 1947.
35. WIDSTROM, G. Biochem. Z. 199 : 298. 1928.
36. WYATT, G. R. Biochem. J. (London), 48 : 581. 1951.

CHEMICAL COMPOSITION OF CEREBRAL ARTERIES

THE CONCENTRATION OF LIPIDS AND MINERALS COMPARED WITH THOSE IN THE INTERNAL CAROTID[1]

By Robert C. Buck, James C. Paterson, and Roger J. Rossiter

Abstract

The concentration of total cholesterol, free cholesterol, total phospholipid, nonlipid phosphorus, calcium, and fat-free dry residue was determined in the internal carotid and cerebral arteries obtained at autopsy from a series of 14 male subjects ranging in age from 40 to 85 yr. From these figures the concentration of cholesterol ester and percentage of the total cholesterol in the ester form was calculated.

In terms of wet weight, there was, in general, no significant difference between the mean concentration of lipids in the internal carotid arteries and that in the cerebral arteries. On the other hand, the mean concentration of calcium and nonlipid phosphorus was much greater in the internal carotid arteries than in the cerebral arteries.

By the method of rank correlation it was found that for both vessels the concentration of total, free, and ester cholesterol was significantly correlated with both age and the severity of the atherosclerosis. Ester cholesterol expressed as a percentage of total cholesterol was also significantly correlated with age and the severity of the disease process.

For the internal carotid artery, the concentration of calcium and nonlipid phosphorus was correlated with both age and the severity of the atherosclerosis. This was in contrast to the cerebral arteries in which the concentration of calcium and nonlipid phosphorus was extremely low and was found to be correlated neither with age nor the severity of the disease process.

Introduction

A number of reports, to be mentioned later in this paper, have indicated that the cerebral arteries differ morphologically from other types of distributing (i.e., muscular-walled) arteries in their normal structure and perhaps also in their manifestations of disease processes. We have been unable, however, to find any reports in the literature in which the chemical composition and chemical changes in the cerebral arteries have been compared with those in a similar type of artery from another location.

The purpose of this paper is to present the results of chemical analyses for lipids and minerals in a series of cerebral arteries and also in a series of internal carotid arteries from the same subjects and to compare the two. The two groups of vessels are compared with regard to alterations in their chemical composition as a result of aging and also of atherosclerosis.

Methods

The arteries were obtained at autopsy from 14 male subjects ranging in age from 40 to 85 yr. The internal carotid artery of each side was divided just below the Circle of Willis, and the vertebral arteries were divided at the level of the foramen magnum. With the brain removed the Circle of Willis,

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the basilar, the anterior, middle, and posterior cerebral arteries and their immediate branches were dissected from the surface of the brain. The entire osseous portion of the internal carotid artery was exposed and as much as possible of the vessel removed. The methods of extraction, separation, and estimation of the lipids were described in a report by Buck and Rossiter (3) and the method of digestion of the fat-free residue and estimation of the minerals was described by Buck (2). Because of the small size of the sample, the individual phospholipids, and the total fatty acids could not be measured.

As the atherosclerotic lesions usually had a patchy distribution, the most severely affected part of the vessel was used as an index of the severity of atherosclerosis in the whole vessel. Such lesions were graded on the basis of their gross appearance into Grades 0, 1, 2, or 3, in a manner similar to that previously described for aortic tissue (3). The specimens of each of the two series of vessels were ranked in order of increasing severity of disease. Using methods of rank correlation, the concentration of the lipids and minerals was investigated in relation to (*a*) the severity of atherosclerosis and (*b*) age of the subject.

Results

Concentration of Lipids and Minerals

Table I shows the concentration of total cholesterol, free cholesterol, cholesterol ester, total phospholipid, calcium, nonlipid phosphorus, and fat-free dry residue in the internal carotid arteries. The mean concentration of each substance, with the standard deviation (S.D.), is shown at the foot of each column. Similar data for the cerebral arteries are given in Table II.

An examination of the mean figures for each set of vessels shows that, except for cholesterol ester, there is no significant difference between the mean concentration of each of the lipids in the cerebral arteries and that of the corresponding lipid in the internal carotid. The concentration of minerals, on the other hand, is much greater in the internal carotid arteries than in the cerebral arteries. The mean concentration of nonlipid phosphorus in the cerebral arteries is only 1/6, and the mean concentration of calcium is less than 1/10 that of the corresponding figure for the internal carotid. In only a single case is the mineral concentration of the same order in both vessels.

Relation to Age and to Severity of Atherosclerosis

In Tables I and II the specimens are arranged in order of increasing severity of atherosclerosis. In each table the concentration of some substances, e.g., total cholesterol, is seen to be greater in the more diseased arteries. It will be noted, however, that there is also a progressive increase in age of the subjects. In Table III the relationship of the concentration of each substance to age and to the severity of artherosclerosis is expressed as the coefficient of correlation (r). The method of rank correlation was used to obtain these coefficients. The significance of the value of r may be judged from its standard error (S.E.r.) and from the probability value (P).

TABLE I

INTERNAL CAROTID ARTERIES: CONCENTRATION OF THE LIPIDS AND MINERALS (MGM./100 MGM. OF WET TISSUE)

(The values are arranged in order of increasing severity of atherosclerosis)

Case number	Age	Total cholesterol	Free cholesterol	Cholesterol ester	Total phospholipid	Calcium	Nonlipid phosphorus	Fat-free dry residue
438	40	0.14	0.13	0.02	0.50	0.143	0.071	21.0
431	47	0.50	0.39	0.19	0.43	0.442	0.139	18.1
463	43	0.22	0.15	0.12	0.52	0.167	0.105	20.6
406	60	0.19	0.14	0.08	0.48	0.131	0.075	14.4
483	69	1.52	0.75	1.30	0.88	0.268	0.130	15.7
346	44	0.23	0.17	0.10	0.55	0.135	0.076	16.9
389	50	0.24	0.18	0.10	0.28	0.392	0.172	19.3
509	61	0.24	0.17	0.12	0.43	0.108	0.258	19.7
493	51	0.26	0.21	0.08	0.50	0.171	0.095	21.1
369	62	1.26	0.90	0.61	1.16	0.763	0.338	18.8
322	61	0.77	0.52	0.42	0.44	0.358	0.162	14.0
425	62	1.44	0.82	1.04	0.72	1.958	1.060	24.6
491	66	1.04	0.54	1.01	0.54	4.990	1.565	27.6
481	85	0.53	0.25	0.47	0.56	0.775	0.314	15.4
Mean	—	0.61	0.38	0.40	0.57	0.772	0.326	19.1
S.D.	—	± 0.48	± 0.27	± 0.41	± 0.21	± 1.261	± 0.422	± 3.7

TABLE II

CEREBRAL ARTERIES: CONCENTRATION OF LIPIDS AND MINERALS (MGM./100 MGM. OF WET TISSUE)

(The values are arranged in order of increasing severity of atherosclerosis)

Case number	Age	Total cholesterol	Free cholesterol	Cholesterol ester	Total phospholipid	Calcium	Nonlipid phosphorus	Fat-free dry residue
463	43	0.23	0.22	0.02	0.68	0.037	0.048	17.8
346	44	0.28	0.27	0.02	0.71	0.000	0.036	13.0
389	50	0.22	0.21	0.02	0.36	0.014	0.040	17.4
431	47	0.18	0.18	0.00	0.68	0.045	0.043	18.7
438	40	0.17	0.17	0.00	0.54	0.025	0.035	16.7
493	51	0.20	0.20	0.00	0.55	0.061	0.037	17.4
406	60	0.18	0.18	0.00	0.60	0.036	0.047	18.7
483	69	0.31	0.27	0.07	0.92	0.093	0.055	18.9
322	61	0.27	0.23	0.07	0.53	0.034	0.043	14.9
491	66	0.70	0.49	0.35	0.77	0.044	0.033	17.4
509	61	0.49	0.39	0.17	0.57	0.376	0.188	19.0
481	85	0.35	0.26	0.15	0.64	0.053	0.036	16.5
369	62	1.25	0.98	0.45	1.09	—	—	17.0
425	62	0.56	0.44	0.20	0.57	0.028	0.039	15.4
Mean	—	0.39	0.32	0.11	0.66	0.065	0.052	17.1
S.D.	—	± 0.29	± 0.27	± 0.14	± 0.17	± 0.092	± 0.040	± 1.6

TABLE III

INTERNAL CAROTID AND CEREBRAL ARTERIES: COEFFICIENTS OF RANK CORRELATION OF THE CONCENTRATION OF LIPIDS, MINERALS, AND FAT-FREE DRY RESIDUE (MGM./100 MGM. OF WET TISSUE) WITH AGE AND WITH THE SEVERITY OF ATHEROSCLEROSIS

	Internal carotid arteries						Cerebral arteries					
	Age			Degree of atherosclerosis			Age			Degree of atherosclerosis		
	r	S.E.*r*	*P*	*r*	S.E.*r*	*P*	*r*	S.E.*r*	*P*	*r*	S.E.*r*	*P*
Total cholesterol	0.81	± 0.10	<0.01	0.64	± 0.16	<0.02	0.75	± 0.12	<0.01	0.74	± 0.13	<0.01
Free cholesterol	0.73	± 0.13	<0.01	0.62	± 0.17	<0.02	0.68	± 0.15	<0.01	0.66	± 0.15	<0.01
Cholesterol ester	0.80	± 0.10	<0.01	0.55	± 0.19	<0.05	0.75	± 0.12	<0.01	0.78	± 0.11	<0.01
Ester cholesterol as % of total cholesterol	0.81	± 0.10	<0.01	0.66	± 0.15	<0.01	0.80	± 0.10	<0.01	0.76	± 0.12	<0.01
Total phospholipid	0.53	± 0.20	<0.05	0.38	± 0.24	>0.1	0.36	± 0.24	>0.1	0.17	± 0.27	>0.1
Fat-free dry residue	− 0.17	± 0.27	>0.1	0.10	± 0.28	>0.1	0.04	± 0.29	>0.1	− 0.13	± 0.27	>0.1
Nonlipid phosphorus	0.72	± 0.13	<0.01	0.80	± 0.10	<0.01	0.06	± 0.29	>0.1	0.06	± 0.29	>0.1
Calcium	0.57	± 0.19	<0.05	0.64	± 0.16	<0.02	0.52	± 0.21	>0.05	0.44	± 0.23	>0.1

In the internal carotid arteries, the concentration of each of the lipids is significantly related both to age and, except for total phospholipid, to the severity of the atherosclerosis. Significant correlations were also found in the ratio of free to ester cholesterol. With increasing severity of atherosclerosis and also with increasing age, ester cholesterol constitutes a progressively larger proportion of the total cholesterol. With the exception of total phospholipid, which is related neither to age nor to the degree of atherosclerosis, the lipids of the cerebral arteries present a similar picture. The concentration of free and total cholesterol, cholesterol ester, and ester cholesterol as a percentage of total cholesterol is correlated with both age and the degree of atherosclerosis. Thus, in this respect, the internal carotid arteries and the cerebral arteries do not differ greatly from the aorta, in which there is an increase in the concentration of certain lipids, particularly cholesterol, with both increasing age and increasing severity of disease process (3).

The relation of the concentration of the nonlipid phosphorus both to age and to the degree of atherosclerosis was found to be surprisingly different in the two vessels. In the internal carotid arteries the coefficient of correlation of the concentration of nonlipid phosphorus with age and with the severity of atherosclerosis is highly significant. In the cerebral arteries, on the other hand, the coefficient of correlation with each of these two variables is only 0.06 ± 0.29.

For calcium the difference between the concentration in the two vessels is less pronounced, but, statistically, the coefficient of correlation with both age and with the severity of the atherosclerosis is not significant for the cerebral and is significant for the internal carotid arteries. It is possible that a study of more vessels might make the correlations for the cerebral arteries significant.

The effect of aging on the concentration of minerals is shown graphically in Fig. 1. The logarithm of the concentration of both calcium and nonlipid phosphorus in the internal carotid artery (points "x") increases with age,

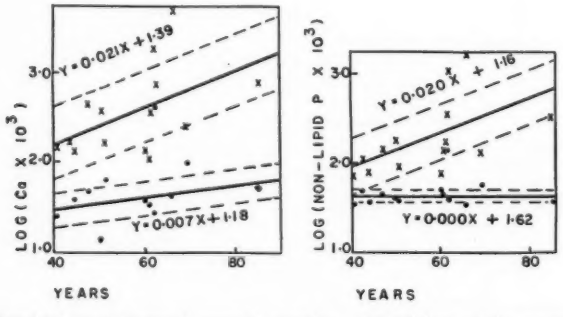

FIG. 1. Cerebral and internal carotid arteries: Logarithm of the concentration of calcium and nonlipid phosphorus in relation to age. The values for the internal carotid artery are represented by the points "x" and for the cerebral arteries by the points "●". The solid line gives the calculated regression line and the broken lines the standard error of estimate of the logarithm of the concentration of the mineral from age.

whereas there is no increase in the logarithm of the concentration of nonlipid phosphorus in the cerebral arteries (points "•") and a comparatively slight increase for calcium. These conclusions are illustrated by the differences in the slopes of the regression lines shown in Fig. 1.

It was previously suggested that in the human aorta the concentration of cholesterol might be considered as a chemical index of the degree of atherosclerosis (3). It was also shown that the logarithm of the concentration of both calcium and nonlipid phosphorus in the human aorta was correlated more closely with both age and the degree of atherosclerosis, as determined by the concentration of total cholesterol, than was the concentration of the mineral itself (2). Fig. 2 shows that the logarithm of the concentration of both calcium and nonlipid phosphorus in the internal carotid artery (points "x") increases with the concentration of total cholesterol, but that the logarithm of the concentration of calcium in the cerebral arteries (points "•") increases only slightly and that of phosphorus not at all.

It may therefore be concluded that, although calcification of the internal carotid artery occurs either in relation to age or to the severity of atherosclerosis, or to both, there is much less change in the mineral content of the cerebral arteries. The concentration of calcium and also of nonlipid phosphorus remains low despite advancing age or increasing severity of the disease. These two processes are, however, associated with a highly significant increase in the concentration of cholesterol in both arteries.

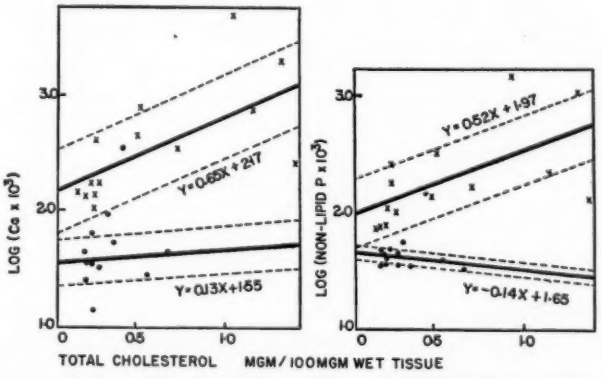

FIG. 2. Cerebral and internal carotid arteries: Logarithm of the concentration of calcium and nonlipid phosphorus in relation to the concentration of total cholesterol. The values for the internal carotid artery are represented by the points "x" and for the cerebral artery "•". The solid line gives the calculated regression line and the broken lines the standard error of estimate of the logarithm of the concentration of the minerals from the total cholesterol concentration.

Discussion

In the human aorta the separate effects of aging and of the pathological process of atherosclerosis on the concentration of lipids (3) and minerals (2) could easily be distinguished. Such a distinction could not so readily be

drawn in the internal carotid and cerebral arteries, but it would appear that the concentration of cholesterol in both vessels and the concentration of calcium and nonlipid phosphorus in the internal carotid artery are significantly related both to aging and to the degree of atherosclerosis.

The low concentration of calcium and nonlipid phosphorus in the cerebral arteries and the poor correlation of the concentration of these substances either with aging or with the degree of atherosclerosis both are of considerable interest. They indicate that calcification of the major branches of the cerebral arteries, when it occurs at all, lags well behind calcification of other muscular-type arteries, in this case one of the arteries from which the cerebral vessels arise.

Calcification in vessels of any type may be a late change in atheromatous intima, a change which can easily be verified by histochemical study of severely diseased arteries. On the other hand, large amounts of calcium are found in the media of arteries, probably as an accompaniment of aging (1, 2, 10, 13). Mineralization of the media may be evident in histological sections as localized deposits and frequently as a replacement of elastic tissue fibers. But, as Buck (2) has shown for the aorta, mineral deposition begins in the early years of life. For a long time it gives no histological evidence of its presence in routinely stained sections. This subtle increase in minerals is again related to aging.

Any attempt to explain the lack of comparability of cerebral arteries to other similar types of arteries would be speculative. However, some of the other ways in which the cerebral arteries are unique might be mentioned, for they may, possibly, have a causal relation to the observations we have made. Anatomically, the cerebral arteries are unique by virtue of their location, not only in relation to the body as a whole, i.e., in a position of minimal hydrostatic pressure, but also in being surrounded by cerebrospinal fluid rather than more solid tissue. According to Hackel (8) and Thoma (12) the internal elastic lamina constitutes most of the elastic tissue of these vessels, the external elastic lamina being absent. The adventitia is rudimentary by comparison with other arteries. Physiologically, these vessels are peculiar in their response to vasoconstrictor influences. Reports by Schmidt (11), by Cobb and Lennox (5), and by Forbes (6) indicate that vasoconstriction in the cerebral arteries is slight by comparison with that in vessels of other organs.

Wells (14) has remarked that the infiltration of lime salts into diseased arteries may serve as a protective mechanism to prevent rupture. The finding of a low concentration of minerals in the cerebral arteries supports this suggestion, since, of the small arteries of the body, they are most prone to rupture.

Finally, it should be mentioned that severe calcification of cerebral vessels does occur in certain specific states, sometimes in association with epilepsy or mental deficiency (7, 9). The choroid plexus is also a frequent site of extensive calcification (4).

Acknowledgments

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References

1. BLUMENTHAL, H. T., LANSING, A. I., and WHEELER, P. A. Am. J. Pathol. 20 : 665. 1944.
2. BUCK, R. C. Arch. Pathol. 51 : 319. 1951.
3. BUCK, R. C. and ROSSITER, R. J. Arch. Pathol. 51 : 224. 1951.
4. CHILDE, A. E. Am. J. Roentgenol. 45 : 523. 1941.
5. COBB, S. and LENNOX, W. G. Federation Proc. 3 : 151. 1944.
6. FORBES, H. S. Arch. Neurol. Psychiat. 43 : 804. 1940.
7. GEYELIN, H. R. and PENFIELD, W. Arch. Neurol. Psychiat. 21 : 1020. 1929.
8. HACKEL, W. M. Virchow's Arch. pathol. Anat. u. Physiol. 266 : 630. 1927.
9. MALLORY, F. B. J. Pathol. Bacteriol. 3 : 110. 1896.
10. RAVAULT, P. Bull. histol. appl. physiol. et pathol. et tech. microscop. 5 : 40. 1928.
11. SCHMIDT, C. F. Federation Proc. 3 : 131. 1944.
12. THOMA, R. Virchow's Arch. pathol. Anat. u. Physiol. 245 : 78. 1923.
13. WEINHOUSE, S. and HIRSCH, E. F. Arch. Pathol. 29 : 31. 1940.
14. WELLS, H. G. *In* Arteriosclerosis—A Survey of the Problem *by* E. V. Cowdry. The MacMillan Co., New York. 1933. Chap. 11.

CYTOCHROME OXIDASE ACTIVITY OF CELL NUCLEI[1]

By David Rubinstein and Orville F. Denstedt

Abstract

The presence of cytochrome oxidase in the nuclear material from chicken erythrocytes and rat-liver cells has been demonstrated with the aid of *p*-phenylenediamine as the agent for reducing cytochrome *c*. The three reagents *p*-phenylenediamine, ascorbate, and hydroquinone are effective in the estimation of cytochrome oxidase in mitochondrial preparations, but only the first mentioned agent can effect the reduction of endogenous cytochrome *c*. The addition of cytochrome *c* will increase the oxidase activity of the liver-cell mitochondria but not of the nuclear fraction from the erythrocytes or the liver cells, presumably because of the impermeability of the nuclear membranes to added cytochrome *c*.

Hogeboom *et al.* (3) have shown that the cytochrome oxidase of the hepatic cell is confined mainly to the mitochondria. The feeble activity detected in nuclear preparations has been attributed to the presence of small amounts of mitrochondria (1, 3). Hogeboom (3) has shown further that there is a definite correlation between the cytochrome oxidase activity of these preparations and the degree of mitochondrial contamination.

The avian erythrocyte, on the other hand, contains a nucleus, but has no mitochondria. According to our studies on the tricarboxylic acid cycle in the chicken erythrocyte all the succinoxidase is contained in the nucleus (5). It may be inferred therefore that cytochrome oxidase also is present in the nucleus.

In the assay of the cytochrome oxidase activity Hogeboom *et al.* (3) used ascorbic acid as the agent for reducing cytochrome *c*. Slater (7), however, has shown that, of all the reducing agents commonly used in these assays, only *p*-phenylenediamine can bring about the reduction of the endogenous cytochrome *c*. Ascorbic acid, hydroquinone, and certain other agents act only on the added cytochrome *c*. This paper describes a further study of the cytochrome oxidase activity of the nuclear and mitochondrial preparations from avian erythrocytes and liver cells, in the presence of hydroquinone, ascorbate, and *p*-phenylenediamine.

Methods

The nuclear material from chicken erythrocytes was prepared as previously described (5). Chicken erythrocytes were suspended in isotonic saline and hemolyzed by freezing and thawing the preparation several times. The nuclear fraction was precipitated by centrifugation and, after isolation, was resuspended in saline. Nuclear and mitochondial preparations were prepared also from rat liver by fractionation with sucrose solutions, according to the procedure of Hogeboom *et al.* (3).

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Contribution from the Department of Biochemistry, McGill University, Montreal, Que.
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The cytochrome oxidase activity of the material was assayed manometrically in the Warburg apparatus. At least three concentrations of the enzyme preparation were used with each determination and the blank value was obtained by extrapolating the plot of the oxygen uptake values to zero enzyme concentration. This procedure compensates for any nonenzymatic oxidation of the reducing agent during the experiment.

Results

The cytochrome oxidase and succinoxidase activities of the nuclear preparation from chicken erythrocytes are indicated in Table I.

The electrons released during the oxidation of succinate to fumarate must pass through the cytochrome oxidase system. Therefore, to be significant, any assay of the cytochrome oxidase activity in an enzyme preparation must be as great as, or greater than the succinoxidase activity of the preparation. However, it may be observed from the results in Table I that, of the three reducing agents used, only *p*-phenylenediamine evoked a significant oxygen

TABLE I

CYTOCHROME OXIDASE AND SUCCINOXIDASE ACTIVITY OF NUCLEAR MATERIAL FROM CHICKEN ERYTHROCYTES

Reducing agent	Without cytochrome *c*	With cytochrome *c* 1.5×10^{-5} *M*	With cytochrome *c* 6×10^{-5} *M*
	μM. O_2 taken up per mgm. N per hour		
p-Phenylenediamine	1.2	1.2	1.4
Ascorbate	0.4	0.4	0.4
Hydroquinone	0.1	0.1	0.1
Succinate	0.8	—	1.0

Flask contents

Hydroquinone.—(After Eichel et al. (2).) Each flask contained 3.0 mgm. hydroquinone in 0.25 ml. H_2O, 0.3 ml. of 0.1 M semicarbazide-hydrochloride (previously adjusted to pH 7.2 with NaOH), 0.4 ml. of 0.5 M phosphate buffer, 0.2 ml. 20% KOH in center well, three levels of enzyme and cytochrome c as indicated above. Volume made up to 3.0 ml. with saline. Gas phase, air. Temp., 37.5°.

Ascorbic acid.—(After Potter (6).) Flasks contained 0.3 ml. of 0.114 M sodium ascorbate, 0.1 ml. of 0.02 M $AlCl_3$, 0.4 ml. of 0.5 M phosphate buffer pH 7.2; enzyme and cytochrome c as above. 0.2 ml. 20% KOH in center well. Volume made up to 3.0 ml. with saline. Gas phase, air. Temp. 37.5°.

p-Phenylenediamine.—Flasks contained 0.6 ml. of 0.15 M pPD, 0.4 ml of 0.5 M phosphate buffer, pH 7.2; enzyme and cytochrome c as above; 0.2 ml. 20% KOH in center well, and saline to make volume up to 3.0 ml. Gas phase, air. Temp., 37.5°.

Succinate.—Flasks contained 0.3 ml. 0.5 M sodium succinate, enzyme and cytochrome c as above; 0.4 ml. of 0.5 M phosphate buffer, 0.2 ml. 20% KOH. Volume made up to 3.0 ml. with saline. Gas phase, air. Temp., 37.5°.

uptake. In all experiments with the nuclear preparations from chicken erythrocytes the addition of cytochrome *c* to the reaction mixtures failed to alter the oxygen uptake.

The cytochrome oxidase activity of the rat liver nuclei and mitochondria, as measured also with the three reducing agents, is indicated in Table II. With both preparations the activity with *p*-phenylenediamine was higher than with ascorbate or hydroquinone.

TABLE II

CYTOCHROME OXIDASE ACTIVITY OF RAT LIVER MITOCHONDRIA AND NUCLEI

Reducing agent	Nuclei		Mitochondria	
	With added cyt.-*c*	Without added cyt.-*c*	With added cyt.-*c*	Without added cyt.-*c*
	μM. O_2 taken up per mgm. N per hour			
p-Phenylenediamine	14.9	8.4	106.8	14.4
Ascorbate	4.5	0.9	75.6	0
Hydroquinone	3.9	0.3	64.8	0
Succinate	16.0	9.2	36.1	14.1

Flask contents: as in Table I. 6×10^{-5} M cytochrome c used when necessary.

Comparing the activity of the nuclear preparations with that of the mitochondrial preparations it may be observed that *p*-phenylenediamine evoked about 3.5 times as great an oxygen uptake in the nuclear preparations as did the other two reducing agents, while in the case of the mitochondrial specimens the uptake was increased by only about a half. Thus the results obtained with the nuclear material from the two sources were qualitatively the same. In the case of the liver cell nuclear preparations the cytochrome oxidase activity obtained with ascorbate, as in Hogeboom's experiments, or with ascorbate or hydroquinone in our study, may be attributed to the presence of mitochondrial residues in the nuclear preparation. Neither ascorbate nor hydroquinone evoked a significant degree of activity in the absence of added cytochrome *c*. A comparison of the cytochrome oxidase activity of the liver mitochondrial and nuclear preparations with various concentrations of added cytochrome *c* may be seen in Figs. 1 and 2 in which the data are presented according to the method of Lineweaver and Burk (4).

With the mitochondrial preparations the cytochrome oxidase activity at maximum cytochrome *c* concentration was the same with the three reducing agents. This is indicated in Fig. 1 by the intersection of the curves on the ordinate. In the case of the nuclear preparation (Fig. 2), the activity obtained with *p*-phenylenediamine was significantly greater than with ascorbate or hydroquinone. These observations support Hogeboom's view

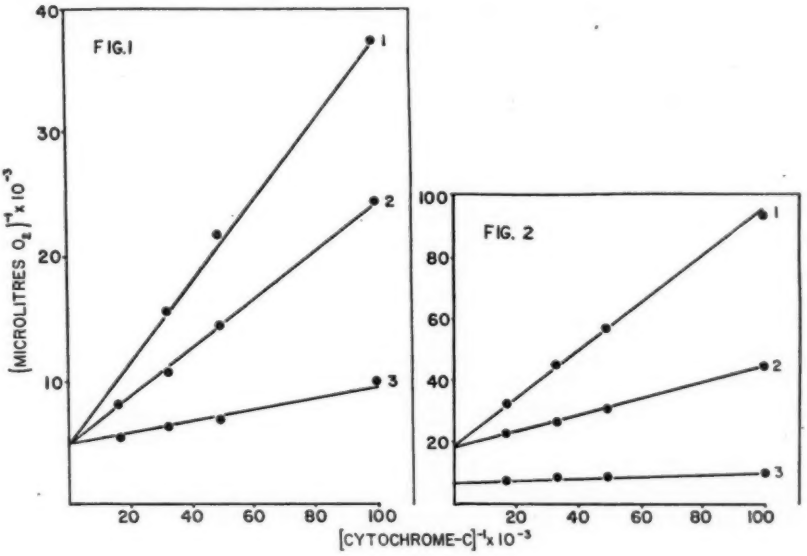

FIG. 1. Effect of cytochrome *c* concentration on the oxygen uptake by liver-cell mitochondria in the presence of reducing agents: (1) hydroquinone, (2) ascorbate, (3) *p*-phenylenediamine. Flask contents as in Table II. Each flask contains the equivalent of 0.1 mgm. mitochondrial nitrogen.

FIG. 2. Effect of cytochrome *c* concentration on the oxygen uptake by liver-cell nuclei in the presence of reducing agents: (1) hydroquinone, (2) ascorbate, (3) *p*-phenylenediamine. Flask contents as in Table II. Each flask contains the equivalent of 0.5 mgm. nuclear nitrogen.

that the activity obtained with ascorbate is attributable to the presence of mitochondria in the preparation. The activity obtained with *p*-phenylenediamine, on the other hand, represents the combined cytochrome oxidase activity of the nuclear material and the contaminating mitochondria. The slope of curve 3 (Fig. 2) indicates that the cytochrome oxidase activity, with *p*-phenylenediamine, is relatively independent of the amount of added cytochrome *c*.

Discussion

From the foregoing discussion it is evident that the nucleus in both the chicken erythrocyte and rat-liver cell contains cytochrome oxidase. It is difficult to prepare nuclear material from rat liver free from mitochondria whereas in the avian erythrocyte the nucleus represents the only particulate material in the cell. When ascorbate or hydroquinone was used as the reducing agent in the assay of cytochrome oxidase the values obtained with the rat liver nuclear preparation were less than those obtained with succinate and therefore were not significant. Any activity that may have been measured, as Hogeboom *et al.* have pointed out and we have confirmed, can be attributed

to the presence of mitochondria in the nuclear material. These two reducing agents do not reduce endogenous cytochrome *c*. With *p*-phenylenediamine, which is capable of reducing endogenous cytochrome, on the other hand, a significant oxygen uptake was demonstrated with the nuclear material from both the chicken erythrocyte and the rat-liver cell and thus the presence of cytochrome oxidase was clearly indicated. The difference between the values obtained with *p*-phenylenediamine and with ascorbate therefore affords an index of the capacity of the nucleus to oxidize reduced cytochrome *c*. The failure of added cytochrome *c* to influence the nuclear cytochrome oxidase activity suggests that the nuclear membranes are not permeable to the cytochrome. In contrast, the mitochondria are permeable to added cytochrome *c*, hence the uniformity of the oxygen uptake with the three reducing agents at the optimal concentration of cytochrome. Since the oxygen uptake obtainable with the nuclear preparation and *p*-phenylenediamine probably depends upon the amount of residual endogenous cytochrome *c* in the material the measured activity may not represent the full cytochrome oxidase activity. In rat-liver and other body-tissue cells by far the greater proportion of the cytochrome oxidase is contained in the mitochondria; that present in the nuclei plays a relatively small role in the total respiratory activity of the cell. In the case of the avian erythrocyte the respiratory activity of the cell is comparatively small but it is maintained entirely by the nucleus.

References

1. DOUNCE, A. L., KAY, E. R. M., and PATE, S. Federation Proc. 12 : 198. 1953.
2. EICHEL, B., WAINIO, W. W., PERSON, P., and COOPERSTEIN, S. J. J. Biol. Chem. 183 : 89. 1950.
3. HOGEBOOM, G. M., SCHNEIDER, W. C., and STRIEBECK, M. S. J Biol. Chem. 196 · 111. 1952.
4. LINEWEAVER, H. and BURK, O. J. Am. Chem. Soc. 56 : 658. 1934.
5. RUBINSTEIN, D. and DENSTEDT, O. F. J. Biol Chem. 204 : 623. 1953.
6. SCHNEIDER, W. C. and POTTER, V. R. J. Biol. Chem. 149 : 217. 1943.
7. SLATER, E. C. Biochem. J. (London), 44 : 305. 1949.

UROPEPSIN AND PLASMA PEPSINOGEN AFTER THE INJECTION OF HISTAMINE DIHYDROCHLORIDE IN DOSES PROVOKING ACUTE GASTRIC ULCERS IN GUINEA PIGS[1]

By Konstanty Kowalewski

Abstract

The effect of an injection of histamine dihydrochloride on the endocrine activity of gastric peptic cells in guinea pigs was investigated. The endocrine activity of the stomach was measured by the determination of an enzyme present in urine (uropepsin) and in the blood (plasma pepsinogen). The dose of histamine injected into the animals in this experiment induced gastric ulcers, found in all sacrificed guinea pigs four hours after the administration of histamine. Uropepsin and plasma pepsinogen were significantly increased in the animals treated with histamine. There appeared to be an analogy between the response to histamine and the results obtained by other writers studing the effect of stress and ACTH on the endocrine activity of the stomach. The possibility is considered that the increase of both uropepsin and pepsinogen in stress or after ACTH is mediated by endogenous histamine.

Introduction

The concept that the peptic cells of the stomach have both endocrine and exocrine functions now seems to be established (2, 3, 15). Uropepsin, an enzyme with proteolytic activity at acid reactions has been found in the urines of man and experimental animals, and it has been concluded that this enzyme is derived from pepsinogen secreted by the gastric cells into the blood and is removed from the blood by the kidneys (15).

A significant correlation seems to exist between the secretion of pepsin in the gastric juice and the excretion of uropepsin. An enzyme responsible for the proteolytic activity of plasma at pH 1.5 was identified as the plasma pepsinogen (16, 17).

As the function of peptic cells of the gastric mucosa is essential for the formation of uropepsin, this enzyme is absent in the urine of gastrectomized animals (1, 4) and patients with achylia gastrica or pernicious anemia (2, 3, 18).

Both uropepsin and plasma pepsinogen are significantly higher in patients with duodenal ulcers than in apparently normal subjects in the comparable age group (17, 18). The plasma of patients with pernicious anemia contains less pepsinogen than that of normal persons of the control group. This finding is so significant that some writers propose the assay of plasma pepsinogen as an aid in the diagnosis of duodenal ulcer and true achylia (17).

The uropepsin excretion appears to be an accurate measurement of gastric pepsin secretion, as shown by the study of the induced hypersecretion of gastric pepsin and acid and a parallel increase of uropepsin (8).

Administration of ACTH or cortisone in man results in an increase of gastric pepsin and uropepsin (8, 20). This response is not altered by vagotomy or

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Contribution from the McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton, Alberta.

atropine (8). These results indicate that the hormonal mechanism plays an important role in the regulation of peptic activity of gastric cells. In some experimental conditions this hormonal mechanism seems to be mediated through the adrenal gland and is intimately associated with the general adaptation syndrome (8).

Some authors suggest that the same hormonal stimuli which provoke the hypersecretion of gastric acid and pepsin may be responsible for the pathogenesis of gastric ulcers in certain experimental conditions and in human beings (8, 20).

Among the metabolites suggested as being responsible for the development of gastrointestinal lesions during the alarm reaction, special attention has been given to histamine (19). On the other side, experimental ulcers of the stomach and duodenum can be easily produced by histamine (9, 13, 14). It is suggested that histamine decreases, in some way, the resistance of gastric mucosa to the proteolytic activity of gastric juice which is rich in pepsin and free acid (13, 14). The vascular factors seem to be responsible for this posthistaminic ulceration of stomachs in experimental animals (13). That this posthistaminic reaction is a purely humoral one may be concluded from the fact that neither vagotomy (5, 6) nor Banthine (unpublished observation) prevents the occurrence of posthistaminic gastric ulcers.

There appears to be a significant analogy in the stomach's response to histamine and to ACTH or stress. Without entering into details of ACTH–histamine relationship (10, 19), it seemed interesting to investigate the effect of histamine on the hormonal activity of peptic cells by the study of posthistaminic changes, if any, in the uropepsin and pepsinogen.

In the present experiment, histamine was injected to the guinea pigs in doses provoking acute gastric ulcers.

Experimental

Thirty male guinea pigs from our own colony, fed on commercial pellets and vegetables, and having a weight range of 430 to 764 gm., were used in this experiment. Twenty animals were selected for the study of plasma pepsinogen and put in separate cages for 24 hr. Only water was allowed them during this period. Ten of these animals were sacrificed without treatment, and the other 10 received subcutaneous injections of Phenergan (Poulenc) in a dosage of 30 mgm. per kilogram of body weight, followed in 30 min. by histamine dihydrochloride (Roche) injected intramuscularly in a dosage of 75 mgm. per kilogram of body weight. As shown previously, the antihistaminic permits the injection of very high doses of histamine as much as 1000 or more times the lethal dose (9, 14). This technique makes it easy to obtain the posthistaminic gastric ulcers in guinea pigs.

The injected animals were sacrificed four hours after the administration of histamine. The blood was collected for the study of plasma pepsinogen. The stomach was removed and the content collected for the determination of free acid. Each stomach was opened along the greater curvature and fixed

on a flat board. It was examined under the dissecting microscope (× 10) so that mucosal lesions could be discovered and charted. The same procedure was used with the treated and nontreated guinea pigs.

As histological studies were done in previous investigations with comparable groups of animals (13), only a few stomachs with ulcers were fixed in Bouin's solution for microscopic examination.

In the remaining 10 animals the study of uropepsin was done a few days before the injection of histamine. The animals were then treated with the same doses of Phenergan and histamine as was used for the study of plasma pepsinogen. The animals were isolated in metabolic cages without food and water for 17 hr., during which time urines were collected, and for seven hours were kept in other cages with food and water ad libitum. This technique of collecting urines in guinea pigs was suggested in a study of urinary corticoids (21).

Plasma pepsinogen was determined according to the procedure of Mirsky *et al.* (16), but plasma was digested with the substrate during 20 hr., and not 24 hr. as in the Mirsky technique.

The hemoglobin substrate method was also used for the study of uropepsin (3) but the urines were dialyzed following the technique described by Duffin and Kowalewski (7).

This dialysis was proved necessary for ridding the urine of interfering chromogenic material (7). Bovine hemoglobin (Wilson Laboratories) was used for the preparation of substrate.

All results are reported in terms of the amount of "tyrosine" released by the proteolytic action of a milliliter of plasma (pepsinogen) or of total urines collected during 17 hr. (uropepsin).

Results

Assays of plasma pepsinogen were performed on 20 guinea pigs, 10 of which have been injected with 75 mgm./kgm. body weight of histamine dihydrochloride. All animals were sacrificed for the study of plasma pepsingogen and gastric content.

Table I presents the results of this experiment. In the normal nontreated animals, the range of values of pepsingogen was from 56 to 120 μgm. per ml. of plasma, and the mean value was 81.0 ± 20 μgm.

The animals treated with histamine and sacrificed four hours after the injection had much higher values of pepsinogen. The range of values in this group was from 250 to 436 μgm. of enzyme, and the mean value was 321.7 ± 49. The results are statistically significant and suggest that the histamine in the dose used in this experiment is a very efficient stimulant for the endocrine activity of peptic cells.

A definite correlation may be observed between the degree of acidity and the plasma pepsinogen of studied animals. The mean free hydrochloric acid of gastric content was 21 ± 5 and 96 ± 15 m.e. per liter, respectively, in normal and treated groups of animals.

TABLE I

EFFECT OF INJECTION OF HISTAMINE DIHYDROCHLORIDE (75 MGM./KGM. BODY WEIGHT) ON THE PLASMA PEPSINOGEN IN THE GUINEA PIGS. PEPSINOGEN EXPRESSED IN MICROGRAMS OF TYROSINE RELEASED PER 1 ML. OF PLASMA AT pH 1.5. FREE HCl OF GASTRIC CONTENT EXPRESSED IN M.E. PER LITER

Normal animals				Animals treated with histamine*			
No.	Body weight in gm.	Free HCl	Pepsinogen	No.	Body weight in gm.	Free HCl	Pepsinogen
1	764	30	120	11	764	88	294
2	615	28	100	12	736	130	436
3	610	16	92	13	736	92	290
4	590	22	70	14	630	104	336
5	590	24	82	15	580	110	360
6	537	18	72	16	553	96	284
7	570	22	74	17	510	98	378
8	500	16	86	18	500	72	265
9	494	18	56	19	495	100	324
10	480	26	58	20	494	70	250
Mean and S.D.	580	21 ± 5	81.0 ± 20	—	599	96 ± 15	321.7 ± 49

* *All guinea pigs of this group presented gastric ulcers four hours after injection of histamine.*

In order to evalute the variability of urinary excretion of uropepsin, the urines were examined during five 17-hr. periods. Histamine was injected immediately before isolation of the animals for the sixth period of collection or urines. The next day no determination was done, but on the eighth day the last assay was performed.

The results are presented in Table II. It is apparent from the review of this table that the daily variations in the excretion of enzyme are not negligible. The mean value for the group is 20.9 mgm. (50 determinations), and the range of individual means (five determinations) is from 17.9 to 22.7 mgm. After the histamine the mean value for the group was 49.6 mgm. with a range from 43.0 to 54.1 mgm. This increase is not as important as the rise of plasma pepsinogen in the comparable experimental conditions, but still seems significant. This conclusion is confirmed by the re-examination of treated animals two days later. At that time the values of uropepsin were comparable to those noted before treatment.

It may be reasonably stated that both plasma pepsinogen and uropepsin increased notably after the injection of histamine. The dose of histamine

TABLE II

EFFECT OF AN INJECTION OF HISTAMINE DIHYDROCHLORIDE (75 MGM./KGM. BODY WEIGHT) ON THE EXCRETION OF UROPEPSIN IN GUINEA PIGS. UROPEPSIN EXPRESSED IN MILLIGRAMS OF TYROSINE RELEASED IN URINE COLLECTED DURING 17 HR.

No. of animal	Body weight, gm.	No treatment						Histamine	No treatment
		Days							
		1	2	3	4	5	Mean of 5 days	6	8
21	615	25.5	28.5	17.3	19.0	23.4	22.7	54.1	23.3
22	612	25.0	22.7	19.1	16.5	20.0	20.0	48.0	19.5
23	570	28.3	19.3	25.8	17.3	18.5	21.8	49.2	18.4
24	566	25.8	22.0	19.5	25.5	18.5	22.2	50.6	26.6
25	566	19.0	19.4	21.1	21.2	17.2	21.6	54.7	28.0
26	550	16.4	15.9	21.0	17.9	18.5	17.9	43.0	17.8
27	480	25.8	18.5	16.6	17.2	23.7	20.4	46.4	25.5
28	480	23.9	22.0	21.4	17.9	18.5	22.4	48.2	19.0
29	453	19.5	19.3	22.7	20.0	16.7	19.6	48.8	23.0
30	430	20.6	19.1	19.5	25.0	19.5	20.7	53.0	18.0
Mean	524	22.7	20.5	20.6	19.7	19.7	20.9	49.6	22.1

(75 mgm./kgm. body weight) was sufficient to provoke the acute gastric ulcers in all animals sacrificed four hours after the injection.

The macroscopical aspects of the gastric mucosa and histological picture of ulcers in guinea pigs were the same as described previously by Kowalewski and Bain (13).

Conclusions

The importance of the dosage in the study of the posthistaminic reaction of gastric mucosa was discussed elsewhere (11, 12). It seems to be established that a direct relationship exists between the dose of histamine and the gastric secretory response (12). The same may be true concerning the endocrine activity of the stomach stimulated by histamine.

In a series of assays (not published) performed in man, treated with 1 mgm. of histamine dihydrochloride (one subcutaneous injection), no significant change was found in the excretion of uropepsin. But when a dose of 4 mgm. was given to the patient protected by an antihistaminic drug (12), a slight rise in the uropepsin was noted.

The dose of histamine given to guinea pigs in this experiment was very high. The endocrine response of stomachs was significant and reflected the

posthistaminic hypersecretion. As may be suspected by the fact of survival and return to apparently good health of all the animals not sacrificed, this ulcerigenic dose of histamine does not kill the animals, and the acute ulcers heal well.

The results obtained approach those described by students of the effect of stress and ACTH on the endocrine activity of gastric mucosa. If one is reminded of the importance of histamine in the general adaptation syndrome and the possibility of hormonal mechanism in the etiology of certain acute and chronic gastric ulcers, one may suppose that some reactions of gastric cells to stress are mediated by histamine.

It also seems probable that histamine plays a role in the production of increased endocrine activity in the stomach after the injection of ACTH or after some stress stimuli, which act even in the absence of the vagus nerves.

References

1. Balfour, D. C., Preston, Fr. W., and Bollman, J. L. Gastroenterology, 10 : 880. 1948.
2. Broh-Kahn, R. H., Podore, C. J., and Mirsky, A. I. J. Clin. Invest. 27 : 825. 1948.
3. Bucher, G. R. Gastroenterology, 8 : 627. 1947.
4. Bucher, G. R. and Ivy, A. Am. J. Physiol. 150 : 415. 1947.
5. Costa, G. and Marconi, R. Boll. mem. soc. piemont. chir. 18 : 1. 1948.
6. Costa, G. and Marconi, R. Minerva chir. 4 : 1. 1949.
7. Duffin, J. D. and Kowalewski, K. J. Lab. Clin. Med. 43 : 163. 1954.
8. Gray, S. J., Ramsey, C., Reifenstein, R. W., and Benson, J. A. Gastroenterology, 25 : 159. 1953.
9. Halpern, B. N. and Martin, J. Compt. rend. soc. biol. 140 : 83. 1946.
10. Karady, S. and Kovacs, A. Nature, 16 : 688. 1948.
11. Kowalewski, K., Conard, V., and Van Geertruyden, J. Compt. rend. soc. biol. 144 : 29. 1950.
12. Kowalewski, K. D. V. A. Treat. Serv. Bull. 8 : 325. 1953.
13. Kowalewski, K. and Bain, G. O. Acta Gastro-Enterologica Belgica. In press. 1954.
14. Marconi, R. and Costa, G. Arch. sci. med. 84 : 1. 1949.
15. Mirsky, A. I., Bloch, S., Osher, G., and Broh-Kahn, R. H. J. Clin. Invest. 27 : 818. 1945.
16. Mirsky, A. I., Futterman, P., and Kaplan, S. J. Lab. Clin. Med. 40 : 188. 1952.
17. Mirsky, A. I., Futterman, P., Kaplan, S., and Broh-Kahn, R. H. J. Lab. Clin. Med. 40 : 17. 1952.
18. Podore, C. J., Broh-Kahn, R. H., and Mirsky, A. I. J. Clin. Invest. 27 : 834. 1948.
19. Selye, H. The physiology and pathology of exposure to stress. Acta Inc., Medical Publishers. Montreal. 1950.
20. Spiro, H. M., Reifenstein, R. W., and Gray, S. J. J. Lab. Clin. Med. 35 : 899. 1950.
21. Zondek, B. and Burstein, S. Endocrinology, 50 : 419. 1952.

CARDIORENAL EFFECTS OF LARGE INFUSIONS OF DEXTRAN IN DOGS[1]

By C. W. Gowdey and I. E. Young

Abstract

The production of hypervolemic dilution anemia in intact, anesthetized dogs by the continuous intravenous infusion of 6% dextran solution caused large increases in the cardiac output and urine flow. No consistent changes were observed in pulse rate or arterial blood pressure. The right auricular mean pressure usually increased early in the infusion, but later there was no consistent relation between right auricular pressure and cardiac output. The total peripheral resistance, glomerular filtration rate, and renal blood flow decreased. With infusion volumes exceeding 10% of the body weight, acute high-output heart failure occurred. The observed hemodilution was consistently greater than that expected from the volume of the infusion, because the dextran solution was, presumably, hypertonic.

Introduction

Most of the investigations of dextran as a plasma substitute have been concerned with plasma volume expansion, excretion, or toxicity. The present paper describes some of the cardiovascular changes produced by infusions of dextran solution into unbled dogs; preliminary reports of this work have been published (4).

Methods

Normal healthy dogs weighing between 10 and 24 kgm. were anesthetized with sodium pentobarbital, and the appropriate catheters and cannulae were inserted. No measurements were made until the animal had been anesthetized for at least two hours. Details of the methods used have been presented in an earlier paper (2). The cardiac output was determined by the direct Fick method, using the Van Slyke technique for analysis of the blood gases. The mixed venous sample was obtained from a catheter in the right ventricle or the pulmonary artery; the arterial sample from a short catheter in the brachial artery. The blood samples were not taken until the dog had been breathing oxygen for at least five minutes, and then were drawn simultaneously over a one-minute period. Oxygen consumption was measured by a Sanborn Metabulator. The mean right auricular and ventricular pressures were determined from catheters connected to saline manometers. The arterial pressure and pulse rate were recorded on a kymograph with a mercury manometer connected to a catheter in the brachial artery. In some cases the auricular, ventricular and/or pulmonary arterial pressures and heart rates were recorded with a Sanborn electromanometer connected to a Smith and Stone oscilloscope-type electrocardiograph. Hematocrit values were determined from arterial blood samples spun in Wintrobe tubes. The glomerular

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filtration rate (GFR) and renal plasma flow (RPF) were determined by measuring the renal clearances of creatinine and sodium *p*-amino hippurate, respectively, over 15-min. periods. An initial priming injection of the test substances was followed by a continuous, slow infusion adjusted to maintain constant plasma levels. The urine was collected from the bladder by an indwelling catheter. Renal blood flow (RBF) was calculated from the RPF and the hematocrit values.

After a suitable control period, dextran solution (Intradex—6% dextran in normal saline) was infused intravenously at a mean rate of 1.0 ± 0.24 (S.D.) ml. per kgm. per min. by a Dale-Schuster pump. The volume infused was recorded every 10 min. Cardiac output, arterial blood pressure, etc., were determined two or three times during the control period and every 30 min. during the infusion.

Results

Fig. 1 shows graphically the changes in cardiac output (CO), mean right auricular pressure (RAP), hematocrit level (Hct), and total peripheral resistance (TPR) in a dog during the continuous infusion of dextran. That part

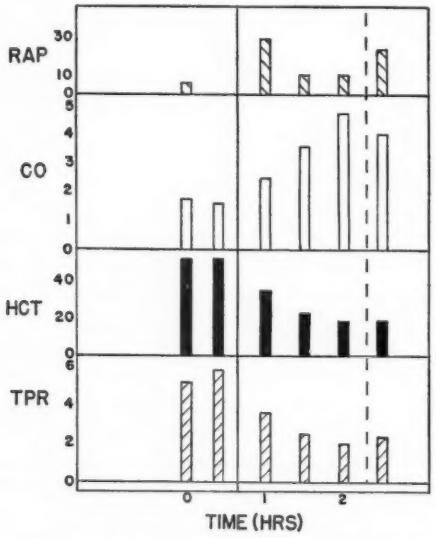

FIG. 1. Cardiovascular effects of dextran infusion; Dog 13 kgm., female. From above downwards: right auricular pressure (RAP) in mm. saline, cardiac output (CO) in liters/min., hematocrit level (Hct) in %, total peripheral resistance (TPR) in small P.R.U's, and time in hours from first control measurements. An intravenous infusion of dextran solution was begun at the vertical line (40 min.) and continued at a steady rate (1.2 ml./kgm./min.) throughout.

Note the large increases in cardiac output as the hematocrit level was reduced by infusion.

The changes depicted in this figure to the left of the vertical broken line are typical of all experiments in this series (except for the RAP—see text). To the right are seen the effects observed in the few experiments in which massive infusions were given.

of the figure to the left of the vertical broken line is typical of this whole series (except for the RAP which was variable); the right side of the figure illustrates the results occurring upon excessive infusion (e.g. in dogs D73, 75, 78). As anemia was produced, reflected by the reduced hematocrit, the cardiac output increased. The mean right auricular pressure at first rose sharply, then fell somewhat, and towards the end of the experiment began to rise again. The total peripheral resistance decreased markedly and remained low throughout the infusion. At the end of the infusion the cardiac output began to fall, while the RAP and TPR increased. This dog received a volume of dextran solution equivalent to 14.6% of its body weight at an average rate of 1.2 ml. per kgm. per min.

Table I is a summary of the changes produced by the infusion of dextran; the table shows the number of the experiment, weight and sex of the dog, time, cardiac output in liters per minute, arterial oxygen transport (cardiac output × arterial oxygen content) in cc. per min., total peripheral resistance—$\frac{\text{blood pressure in mm. Hg}}{\text{cardiac output in ml. per sec.}}$, right auricular mean pressure in mm. saline, volume of dextran infused as a percentage of the body weight, and the urine flow, glomerular filtration rate, and renal blood flow, in ml. per min.

With the infusion of dextran the hematocrit level decreased markedly, and the cardiac output invariably increased; in some experiments (e.g. D 73, 75, 78, 80, 83, 84, 91) the output increased by more than 100% over control levels. Usually the cardiac output continued to rise as infusion progressed until a volume equivalent to about 10% of the body weight had been given; with further infusion high-output failure suddenly developed (D 73, 75, 78), and the animal died within a few minutes. Cardiac failure is here defined as that state in which the elevated cardiac output has begun to fall, and continues to fall, while the right auricular mean pressure rises abruptly. The rapidity with which failure developed is illustrated in experiment D 73. The highest cardiac output measured was at 4.00 p.m.; 30 min. later the output had fallen slightly, and the RAP had risen precipitously; death occurred 10 min. later. Gross examination post mortem usually revealed a dilated heart, distended veins, engorged liver, and often ascites and pulmonary edema; pitting edema of the extremities and petechial hemorrhages in the intestine and kidneys were occasionally seen.

The arterial oxygen transport was usually maintained during the early stages of infusion, the increasing cardiac output compensating for the decreasing arterial oxygen content. When the hematocrit level had fallen below 20%, however, the arterial oxygen transport was reduced, and when cardiac failure occurred, it was markedly reduced. The arteriovenous oxygen difference grew progressively smaller throughout the infusion.

The arterial blood pressure either did not change appreciably, or rose with the infusion; in no experiment did the mean pressure fall below 90 mm. Hg before the onset of frank heart failure. The total peripheral resistance invariably decreased, often to less than 50% of the control values; however, when

TABLE I

SUMMARY OF EFFECTS OF DEXTRAN HYPERVOLEMIA

Dog Wt. Sex	Time	Hct. %	Cardiac output (liters/min.)	Art. O_2 trans. (cc./min.)	TPR	RAP (mm. saline)	Vol. inf. (% b. wt.)	Urine flow (ml./min.)	GFR (ml./min.)	RBF (ml./min.)
No. 69	12:15	55.0	1.80	420	4.86	70	—	0.77	72	490
	1:35	51.0	1.96	460	4.22	55	—	—	—	—
23.6 kgm.	1:55	Infusion begun								
Fem.	2:55	31.0	3.68	401	2.34	370	5.0	2.3	46	715
No. 70	12:55	44.0	1.96	384	4.96	48	—	—	—	—
	1:20	46.0	1.76	345	5.40	50	—	0.3	56	245
18.1 kgm.	1:35	Infusion begun								
Fem.	2:10	38.0	2.28	340	4.05	126	1.2	0.20	46	235
	2:40	29.0	2.61	286	3.46	172	3.6	0.37	42	203
	3:25	24.0	3.35	193	3.04	255	6.1	0.77	44	310
No. 71	12:30	50.0	2.02	380	3.78	16	—			
	1:40	Infusion begun								
13.0 kgm.	2:10	22.0	2.64	249	3.00	230	5.5			
Fem.	2:37	14.0	3.02	182	2.94	201	9.6			
No. 73	12:00	52.0	1.60	370	5.16	2	—	0.5		
	1:00	53.0	1.26	288	6.18	20	—	0.6		
13.0 kgm.	1:30	Infusion begun								
Fem.	2:00	39.0	1.86	319	4.62	101	2.6	0.7		
	2:30	28.0	3.11	396	3.06	70	5.1	2.7		
	3:00	24.0	3.00	313	3.06	45	7.5	2.1		
	3:30	20.0	3.07	258	2.88	80	10.0	1.9		
	4:00	15.0	3.21	197	2.64	120	12.5	2.1		
	4:30	13.0	2.86	113	2.82	384	16.2	0.7		
	4:40	DIED								
No. 75	12:05	54.0	2.17	524	3.84	26	—	0.39	62	365
	1:25	55.0	1.76	430	4.56	45	—	0.13	73	328
15.0 kgm.	1:35	Infusion begun								
Fem.	2:05	53.0	2.76	475	3.06	25	2.9	—		
	2:35	31.0	4.61	580	1.86	70	5.9	0.37	22	210
	3:05	26.0	3.39	374	2.40	110	7.5	Trace	35	223
	3:35	27.0	4.46	382	1.62	123	11.8	—	32	189
	4:05	23.0	3.94	218	1.86	150	14.5	—	15	58

TABLE I—*Continued*

SUMMARY OF EFFECTS OF DEXTRAN HYPERVOLEMIA—*Continued*

Dog Wt. Sex	Time	Hct. %	Cardiac output (liters/min.)	Art. O_2 trans. (cc./min.)	TPR	RAP (mm. saline)	Vol. inf. (% b. wt.)	Urine flow (ml./min.)	GFR (ml./min.)	RBF (ml./min.)
No. 78	12:55	50.0	1.70	376	5.10	5	—	0.8		
	1:20	50.0	1.58	329	5.76	—	—	1.2		
13.0 kgm.	1:33	Infusion begun								
Fem.	1:55	34.0	2.49	374	3.60	28	2.5			
	2:25	22.0	3.53	336	2.52	10	6.8	3.5		
	2:55	18.0	4.66	370	1.98	10	10.5	—		
	3:25	18.0	3.97	257	2.22	23	14.6	3.6		
No. 79	1:25	34.0	2.52	339	3.06	14	—	1.5	50	135
	1:55	34.0	3.13	375	2.52	14	—	1.8	62	147
13.0 kgm.	2:30	Infusion begun								
Fem.	3:09	26.0	3.63	332	2.34	136	2.8	1.9	45	116
	3:45	11.0	5.60	304	1.20	0	7.2	3.3	69	220
	4:25	9.0	3.28	147	2.10	0	10.5	0.3	13	61
No. 80	3:50	37.0	1.96	318	3.00	0	—	0.74	54	250
	3:51	Infusion begun								
15.5 kgm.	4:40	20.0	2.25	211	3.20	13	2.8	0.30	44	196
Male	5:10	14.0	2.68	175	2.87	15	4.9	0.47	37	210
	5:50	9.0	4.26	171	1.63	117	9.0			
No. 81	1:02	39.0	2.68	460	2.88	13	—	1.1	66	230
	1:32	39.0	2.10	350	3.60	13	—	1.4	63	218
12.3 kgm.	1:46	Infusion begun								
Fem.	2:16	23.0	2.17	241	3.96	15	3.2	2.4	57	244
	2:48	15.0	3.06	227	2.88	39	6.4	2.5	51	223
	3:16	12.0	3.94	216	2.40	65	8.0	2.8	48	189
	3:41	9.0	3.79	146	2.34	104	11.5	1.6	42	200
No. 82	12:05	47.0	1.33	270	6.24	—	—	2.5	49	198
	12:55	44.0	1.26	236	6.48	13	—	1.8	43	—
12.3 kgm.	1:10	Infusion begun								
Fem.	1:40	30.0	2.49	273	4.08	130	2.9	3.1	47	159
	2:10	19.0	1.70	156	5.40	208	5.7	3.9	56	209
	2:40	15.0	2.47	146	3.60	247	8.8	1.8	38	184

TABLE I—*Concluded*

SUMMARY OF EFFECTS OF DEXTRAN HYPERVOLEMIA—*Concluded*

Dog Wt. Sex	Time	Hct. %	Cardiac output (liters/min.)	Art. O_2 trans. (cc./min.)	TPR	RAP (mm. saline)	Vol. inf. (% b. wt.)	Urine flow (ml./min.)	GFR (ml./min.)	RBF (ml./min.)
No. 83	12:59	48.0	1.74	380	—	39	—	0.7	64	430
	1:31	49.0	1.61	350	4.32	36	—			
18.0 kgm.	2:00	Infusion begun								
Male	2:36	30.0	3.84	518	1.74	—	3.2	0.9	62	398
	3:04	20.0	3.96	378	—	—	6.3	1.1	55	288
	3:45	15.0	4.49	294	1.92	—	11.7	0.9	68	288
	4:07	12.0	4.39	259	1.98	338	13.3	Trace	11	264
No. 84	12:35	54.0	2.57	526	—	—				
	1:25	56.0	2.49	517	—	− 13	—	1.0	62	340
19.0 kgm.	1:48	Infusion begun								
Male	2:20	29.5	4.44	526	—	− 13	3.4	0.5	80	362
	2:50	21.0	4.70	394	—	—	5.9	0.4	31	—
	3:05	15.5	5.31	317	—	91	7.7	0.5	40	174
No. 91	11:10	52.0	2.10	418	2.62	—	—	0.5	91	610
	11:50	52.0	3.12	590	1.72	− 65	—	0.7	75	600
15.4 kgm.	12:10	Infusion begun								
Fem.	12:40	34.0	2.91	469	2.64	− 26	4.0			
	1:20	24.5	5.52	568	1.30	52	9.2	2.0	45	540
No. 92	11:45	48.5	3.04	494	2.96	—	—	3.2	66	370
	12:25	Infusion begun								
18.8 kgm.	12:55	29.5	3.28	362	—	—	2.7	8.4	64	415
Fem.	1:25	20.0	4.47	338	—	—	5.0	9.7	62	328
No. 93	11:40	50.0	2.45	452	2.62	—	—	0.9	47	332
	12:40	51.1	1.98	572	2.80	65	—	1.0	56	330
16.3 kgm.	1:05	Infusion begun								
Fem.	1:40	28.0	3.28	380	2.48	247	4.1	1.9	53	250
	2:10	19.0	4.23	336	—	321	6.7	2.8	43	370
	2:45	—	—	—	—	—	—	5.5	42	—

the cardiac output ceased to rise, the total peripheral resistance began to increase. The pulse rate showed no consistent response to infusion, but usually decreased with cardiac failure.

The right auricular mean pressure increased with infusion, but in some experiments (e.g. D 73, 78, 79) after rising sharply at the beginning, it decreased towards control levels. During the later stages of infusion it tended to rise. With cardiac failure imminent, the RAP increased very rapidly (e.g. D 73, 75, 80, 81). The right ventricular and pulmonary arterial pressures followed the same general pattern.

Progressive decreases in glomerular filtration rate, and usually in renal blood flow were produced by the infusion; the ratio of renal blood flow to cardiac output consistently fell (down to 50% of control). Dextran infusion invariably caused a diuresis, but when cardiac failure occurred, there was a marked decrease in urine flow and, occasionally, anuria.

Discussion and Conclusions

Although the cardiac output always increased as anemia was produced by the infusion (Table I), the changes in right auricular pressure were not consistent. Any direct relation between cardiac output and right auricular pressure is, therefore, not evident in these experiments. In other experiments (5, 8) acute anemia was produced by removing one-half of the calculated blood volume and simultaneously replacing it by dextran solution. Large increases in cardiac output occurred although the degree of hypervolemia was much less (*vide infra*) than in the present series. Thus the importance of the arteria oxygen content in regulating the cardiac output is apparent.

The glomerular filtration rate and renal blood flow usually decreased as the cardiac output rose; the ratio of renal blood flow to cardiac output was, therefore, markedly decreased (down to 50% of control). This is evidence that renal vasoconstr ction caused a redistribution of blood, probably to other vascular beds which are more sensitive to hypoxia. Earlier experiments with gum acacia infusions (2) showed that part of the increased cardiac output went to the muscle and skin areas. Two factors may be involved in the renal vasoconstriction: hypervolemia itself and anemia. Although these factors cannot be separated in the present experiments, other investigators have observed a decreased renal blood flow both in severe anemia in patients (1) and in experimentally-produced hemorrhagic anemia in dogs (7). Low renal blood flows were obtained during the control periods in the low-hematocrit dogs in the present series (D 79, 80, 81).

It was observed that the reductions in hematocrit levels in these experiments were always greater than could be accounted for by the volumes of dextran solution infused. This was true even if it were assumed that all of the infusion remained within the cardiovascular system. That the smaller dextran molecules were readily excreted in the urine was shown by a positive anthrone test for dextran. The consistently observed diuresis can be explained by these osmotically-active dextran molecules in the glomerular filtrate. Renal

excretion of dextran has previously been reported by Wasserman and Mayerson (9), who also demonstrated the rapid appearance of dextran in the lymphatic system. Thus if the dextran solution had been iso-osmotic, the plasma expansion (decrease in hematocrit level) should have been less instead of more than the volume infused. Recent studies in this laboratory (6) of the mean corpuscular hemoglobin concentrations during dextran infusions indicate that the Intradex used had a markedly higher effective osmotic pressure than that of dog plasma. This explains the unexpectedly large decreases in hematocrit levels obtained in these experiments.

In these studies it again (3, 2*b*) appears that changes in the cardiac output may be independent of the changes in the right auricular pressure; however, there is a direct correlation between the increases in cardiac output and the degree of anemia. The presence of a decreased renal blood flow in this hyperdynamic hypervolemic state may also be evidence of the profound influence of reduced oxygen transport in cardiovascular regulation.

The most striking feature of the cardiac failure produced by dextran infusion was the rapidity of onset and the short survival time. Unlike that produced by gum acacia solution (3, 2) this failure was often accompanied by frothy pulmonary edema. Even though the volumes of dextran solution used in these experiments were very large, it is of interest that acute heart failure did occur in dogs with previously normal hearts (normal ECG, cardiac output) when the anemia became severe. Hypervolemia, especially when produced by non-oxygen-carrying plasma expanders, is potentially dangerous, perhaps imminently so in patients with impaired cardiac function.

Acknowledgments

We wish to acknowledge our debt to Prof. J. A. F. Stevenson for his general criticisms and advice, and, in particular, one of us (I.E.Y.) wishes to express her appreciation for his direction in the renal studies. Dr. J. E. Merriman taught us the techniques of pulmonary artery catheterization and phasic pressure recording. Mr. R. C. Payson gave valuable technical assistance throughout the study. The Intradex used in these experiments was generously supplied by Mr. L. Gullick of Glaxo (Canada) Limited.

References

1. Bradley, E. W. and Bradley, G. P. Blood 2 : 192. 1947.
2. (*a*) Gowdey, C. W., Hatcher, J. D., and Sunahara, F. A. Rev. can. biol. 10 : 70. 1951. J. Pharmacol. Exptl. Therap. 101 : 15. 1951.
   (*b*) Gowdey, C. W., Hatcher, J. D., and Sunahara, F. A. Can. J. Biochem. Physiol. 32 : 282. 1954.
3. Gowdey, C. W. and Loynes, J. S. Can. J. Med. Sci. 30 : 314. 1952.
4. (*a*) Gowdey, C. W. and Young, I. E. Proc. Can. Physiol. Soc. 16 : 28. 1952. Rev. can. biol. 13 : 64. 1954.
   (*b*) Gowdey, C. W. and Young, I. E. Federation Proc. 12 : 324. 1953.
5. Merriman, J. E., Sunahara, F. A., Young, I. E., Gowdey, C. W., and Stevenson, J. A. F. Proc. Can. Physiol. Soc. 16 : 43. 1952. Rev. can. biol. 13 : 75. 1954.
6. Merriman, J. E., Young, I. E., and Gowdey, C. W. Federation Proc. 13 : 387. 1954.
7. Patterson, J. C. S. Am. J. Physiol. 164 : 682. 1951.
8. Sunahara, F. A., Merriman, J. E., and Gowdey, C. W. Federation Proc. 12 : 369. 1953.
9. Wasserman, K. and Mayerson, H. S. Am. J. Physiol. 171 : 218. 1952.

A RAPID METHOD FOR FILTER PAPER ELECTROPHORESIS[1]

By S. D. Vesselinovitch and H. S. Funnell

Abstract

A technique for filter paper electrophoresis and strip staining that permits the running time to be reduced to two hours or less is described. Some typical two hour electrophoretic patterns are illustrated and the clinical advantages of the technique mentioned. Details of a simple and inexpensive apparatus for electrophoresis on filter paper are given. Several variables affecting rapid and distinct protein separation, which were modified in the development of this method, and the relationships between these changes and the shortened running time are discussed.

Introduction

Numerous clinical-laboratory tests have been developed for the detection of quantitative and qualitative changes occurring amongst the plasma proteins during disease. Of these, filter paper electrophoresis, which was independently introduced by several different workers in 1950 (1, 3, 8), seems to be the most effective and extensively used.

Modifications of the original techniques along several different lines have done much to improve this method (4, 5, 6, 7). However, such procedures still have the major disadvantage from the clinical viewpoint, of taking from 5 to 18 hr. running time. Furthermore, despite an extensive literature, no clear-cut standardization of procedure seems to have emerged to date. This report is concerned with the development of a rapid (one-half to two hour) method and the establishment of a standardized procedure for such short runs, relating time to such other variables as potential gradient, ionic strength of buffer, and dimensions of strip.

Materials

The electrophoretic chamber (Fig. 1) consists of a plastic refrigerator crisper (32 × 23× 10 cm.) within which is placed a second plastic container (30 × 20 × 4 cm.) divided lengthwise into four equal sections (*A*). The compartments of this latter container hold the buffer. Platinum foil electrodes (*B*) are fixed to the two central compartments while a bridge of facial tissue (*C*) connects each of these to the outer vessels. A nylon brush support (*D*) is utilized (2). This was made of a rigid plastic plate (27 × 15 cm.) into which were cemented nylon bristles at 15 mm. intervals. These bristles project 5 mm. above the upper surface of the plate. This paper support is held in position by two parallel rods (*E*) fixed 2 cm. in and 5 cm. down from either side of the main chamber to allow for its easy removal. A capillary micropipette with 0.005 ml. divisions was used for serum applications.

A well filtered full wave rectifier with a range of 0 to 1000 volts was especially designed as the power supply for this work.

[1] *Manuscript received May 10, 1954.*
Contribution from the Ontario Veterinary College, Guelph, Canada. This work was supported in part by a grant-in-aid from the National Cancer Institute of Canada.

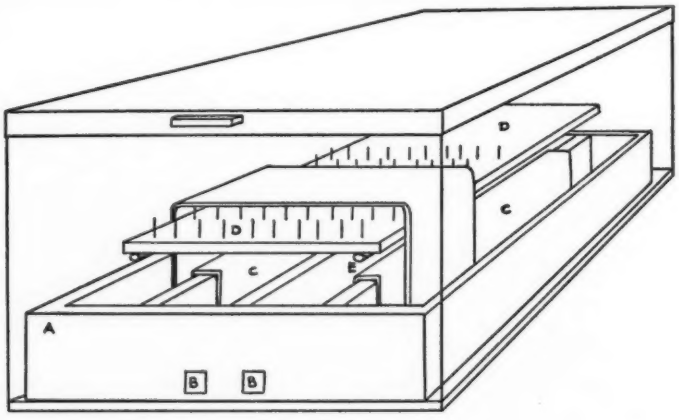

FIG. 1. Electrophoretic chamber.
A—buffer container; B—electrodes; C—facial tissue bridge; D—nylon brush support; E—supporting rod.

Procedure for a Two Hour Run

Strips of Whatman No. 1 filter paper (28 × 4 cm.) are saturated with barbiturate buffer, pH 8.6, with an ionic strength of 0.05. The excess buffer is removed by laying the strips on a paper towel for a few seconds (until the shine is lost) before they are placed on the nylon brush support with their ends hanging free. The support, which should be kept horizontal, is transferred to the chamber so that the overhanging ends of the strips are submerged a few millimeters into the buffer in the side vessels.

Serum (0.005–0.01 ml.) is now evenly applied, by means of the micropipette, along a line across the strip 1.5 cm. from its center towards the negative pole. The chamber is then closed and the current (potential gradient 12–14 v./cm.) is switched on for a period of two hours, after which time the strips are removed and dried for 10 min. in an oven at 120° C.

The staining method used is a modification of the technique of Durrum (3). Dried strips are first stained for five minutes with 1% bromphenol blue in ethanol saturated with mercuric chloride. The strips are washed by immersion in repeated changes of a 0.2% acetic acid solution until such time as no further trace of yellow color appears in the wash. This results in a clear white background against which the protein fractions are dyed a faint yellow. Finally, immersion of these faintly stained strips in absolute ethyl alcohol for two minutes will convert the yellow to an intense blue. The marked contrast thereby achieved between the white background and the intensely colored protein fractions is of particular value for naked eye interpretation of the strip.

Fig. 2 presents several electrophoretic patterns of normal and pathological sera which were run for two hours under the above conditions.

PLATE I

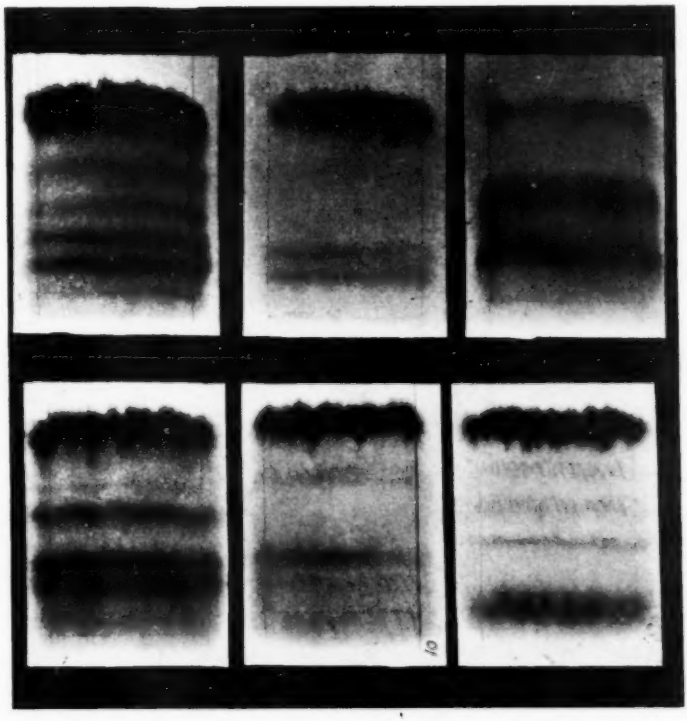

FIG. 2. Electrophoretic patterns of five canine and one human serum (bottom right pattern). The classical components in each pattern are readily recognized from top to bottom as albumen, α_1, α_2, β, and γ-globulins. The actual dimensions of these strips were 28 × 4 cm.

Discussion

The apparatus and procedure described above have several advantages and features that particularly suit them for rapid clinical laboratory use. The chamber is inexpensive and easily assembled, while the removable support makes strip setting simple.

The flow of electric current through the strip results in its becoming heated with a consequent loss of moisture. Such heating and evaporation increases with the amount of contact between the paper strip and its supporting surface, thereby causing increased degrees of diffusion, endo-osmosis, and electro-osmosis. This type of interference with the electrophoretic separation of the proteins is considerably reduced by the use of a nylon brush support.

Whatman No. 1 filter paper was found to give clearer, more sharply separated protein components than did the heavier and rougher Whatman No. 3. Strips ranging from 10 to 40 cm. long and from 2.5 to 4 cm. wide were tested; those measuring from 26 to 34 cm. × 4 cm. proved most satisfactory. Shorter strips allow too much interference from endo-osmosis and electro-osmosis, while longer ones, requiring higher voltage, are liable to be adversely affected by increased heat.

Buffer with an ionic strength of 0.05, as opposed to the more commonly used 0.1, was preferred because it increased the mobility of the protein fractions. However, further reduction (0.025) resulted in indistinct separations.

Because the degree of separation achieved between two boundaries is to a considerable extent dependent on the total quantity of protein present, the use of a very small and evenly distributed amount of serum (0.005 to 0.01 ml.) has the same advantage as that attributed to dilution in free electrophoresis. Large quantities of sera with a short running time result in overcrowding of the proteins causing an overlapping of the bands.

During the course of the numerous trials undertaken in our attempt to develop a rapid filter paper electrophoresis technique, the optimal values of potential gradients for times ranging from one-half to five hours are established. This is summarized in Table I. It is apparent that a definite inverse relationship existed between time on the one hand and potential gradient on the other.

TABLE I

Time, hr.	Potential gradient v./cm.
½	18–21
1	15–17
2	12–14
3	9–10
4	8– 9
5	6– 7

One-half and one hour runs resulted in protein separations comparable to those achieved at two hours. However, at the shorter times, naked eye readings of the electrophoretic patterns were not possible.

The use of a higher potential gradient than that shown in Table I resulted in overheating of the strip, producing drying and subsequent scorching of the paper. Conversely, at potential gradients of 14 v./cm. and higher, any prolongation of times beyond those shown in Table I resulted in indistinct separations. This apparently results from conflicting migratory effects of the endo-osmotic and electro-osmotic flows on the precipitating proteins.

Acknowledgment

The power supply used in this work was designed by Prof. J. G. F. Morton, Department of Physics, Ontario Agricultural College, Guelph, Ont., whose interest and assistance in this problem are gratefully acknowledged. The assistance of Dr. J. P. W. Gilman is also acknowledged for his constructive help in preparing this manuscript.

References

1. CREMER, H. D. and TISELIUS, A. Biochem. Z. 320 : 273. 1950.
2. DETTKER, A. and ANDUREN, H. Private communication.
3. DURRUM, E. L. J. Am. Chem. Soc. 72 : 2943. 1950.
4. FLYNN, F. V. and DE MAYO, P. Lancet, 261 : 235. 1951.
5. GRASSMANN, W., HANNIG, K., and KNEDEL, M. Deut. med. Wochschr. 76 : 333. 1951.
6. GRIFFITHS, L. L. J. Lab. Clin. Med. 41 : 188. 1953.
7. MCDONALD, H. J., URBIN, M. C., and WILLIAMSON, M. B. J. Colloid Sci. 6 : 236. 1951.
8. TURBA, F. and ENENKEL, H. J. Naturwissenschaften, 37 : 93. 1950.

LECITHINASE SYSTEMS IN SUGAR BEET, SPINACH, CABBAGE, AND CARROT [1,2]

By Morris Kates

Abstract

Lecithinase activity of aqueous extracts of sugar beet, spinach, or cabbage leaves, and of carrot root was found to be associated entirely with the plastid fractions, separated by high-speed centrifugation. The supernatant cell sap–cytoplasm fractions were not only inactive but actually inhibitory. The rate of enzymatic liberation of choline from lecithin by all plastid fractions was found to be greatly increased by saturation with diethyl ether. The influence of pH, enzyme concentration, substrate concentration, and temperature on the rate of ether-activated choline liberation was studied and optimum conditions for the reaction were determined. Under optimum conditions, liberation of choline from lecithin by each of the plastid fractions was rapid and was accompanied by a much slower liberation of inorganic and water-soluble organic phosphate; liberation of phosphates was much greater with spinach than with the other species. Thermal inactivation and fluoride inhibition of the enzyme systems were also studied.

Introduction

Investigation of the enzymatic hydrolysis of phospholipids by plant tissues has been greatly stimulated by the recent findings of Hanahan and Chaikoff (11, 12). These investigators have shown that aqueous extracts of fresh carrot root or cabbage leaves are capable of enzymatically hydrolyzing glycerophosphatides at their nitrogenous base-phosphoric acid linkages. These findings have in general been confirmed by the work of Rose (20) with cabbage and of Acker, Diemair, and Jäger (1) with carrot, and the widespread occurrence in plants of such a phospholipid-splitting enzyme has been established through the extensive studies of Ducet (7). Hanahan and Chaikoff observed little or no formation of free fatty acids or inorganic phosphate from their substrate. Ducet (7), however, concluded, through indirect evidence, that enzymes liberating fatty acids from phospholipids are present in many plants, and Acker, Diemair, and Jäger (1) and Holden (14) have reported the formation of inorganic phosphate from phospholipids by carrot root press juice, and by tobacco leaf chloroplasts, respectively. Lecithinase (choline-liberating) activity has recently been demonstrated in latex serum of *Hevea brasiliensis* (21).

In this laboratory, rapid liberation of choline was observed when spinach chloroplasts were incubated with pure lecithin in the presence of diethyl ether (16). A systematic study of lecithinases in extracts of several plant species was then undertaken to determine the intracellular location of these enzymes and to establish optimum conditions for their activity in the presence of ether. The results of this study are presented here in detail.

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada. Issued as N.R.C. No. 3533.

[2] *Presented in part at the meeting of the American Society of Plant Physiologists, Cornell University, Ithaca, N.Y., September 7, 1952.*

Materials and Methods

Plant Sources

Sugar beet and cabbage were grown in the greenhouse; carrot was obtained on the market. Spinach was obtained from both sources.

Preparation of Enzymic Material

Leaf extracts were prepared by blending 200 gm. of fresh, washed, and blotted leaves with 160–170 ml. of distilled water in a chilled Waring blendor for three or four minutes, squeezing the *brei* through nylon cloth, and centrifuging the juice for one minute at 1800 × *g* to remove whole cells and debris. The supernatant leaf extract was separated, by centrifugation at 15,000 × *g* for 20–25 min., into a chloroplast fraction (dark green sediment) and a cell sap – cytoplasm fraction (yellowish green supernatant). The chloroplast fraction was suspended in distilled water (80 ml.), recentrifuged at 15,000 × *g* and resuspended in distilled water (35 to 45 ml.) to give a suspension containing 2.5–3.5 mgm. of chlorophyll and 35–50 mgm. of dry weight per ml.

Carrot root extracts were prepared and fractionated in the same way as the leaf extracts, using 200 gm. of freshly scraped, washed, and sliced carrot root. The orange sediment (chromoplast fraction) obtained by high-speed centrifugation of the carrot root extract was washed once with distilled water, recentrifuged, and resuspended in distilled water to a volume of 20–25 ml. (to contain 20–25 mgm. dry weight per ml. suspension).

All operations were carried out in a cold room at 4°–5° C. The enzyme preparations were kept at 5° C., and showed only slight losses in original activity after 10 days. Preparations older than one week were not used.

Since no attempt was made to control the pH or osmotic pressure of the blending medium, the plastid fractions obtained by high-speed centrifugation would be expected to consist mostly of chloroplast or chromoplast fragments contaminated, however, with some nuclear and mitochondrial material (see Weier (23)). Thus, the terms chloroplast or chromoplast fractions used here refer to mixtures of particulate material and not to intact, uncontaminated plastids.

Substrate

The substrate used in this work was phosphatidyl choline (lecithin), prepared from egg yolk as described by Hanahan, Turner, and Jayko (13). Analysis* showed that the lecithin preparation contained essentially all of its nitrogen in the form of esterified choline. The substrate was stored at 5° in methanolic solution. When required, a suitable aliquot was brought to dryness *in vacuo* (nitrogen stream) and the dried, weighed residue was made up to a 5% (w/v) emulsion with distilled water.

Analytical Methods

Phosphorus was determined by the method of King (17), total nitrogen by the micro-Kjeldahl method, and iodine number according to Yasuda (25).

* *The substrate had 4.08% P, 1.88% N, 15.8% choline; N/P atomic ratio 1.01, choline/P molar ratio, 0.99; 70.0% fatty acids (iodine number 71.5, neutral equivalent 292); iodine number of lecithin 59.2; and* $[\alpha]_D^{25} + 6.7°$ *(c, 3.84 in chloroform–methanol, 1 : 1).*

Free choline was determined by a combination of the methods of Entenman, Taurog, and Chaikoff (8) and of Glick (9), as follows: 10 ml. of aqueous solution containing 0.5 mgm. to 4.0 mgm. of free choline was placed in a 15 ml. graduated centrifuge tube and made alkaline to phenolphthalein with 1 *N* sodium hydroxide; 5 ml. of a 2% methanolic solution of ammonium reineckate was added and the mixture was stirred with a glass rod and kept at 5° C. for two hours. The precipitate was separated by centrifugation and was washed twice in the centrifuge tube with 2 ml. portions of *n*-propanol. The choline reineckate crystals were then dissolved in acetone (8 ml.), and the solution was cleared by centrifugation, decanted into a 10 ml. volumetric flask, and made up to volume with acetone. The absorbance of the solution at 526 mμ was read with a Coleman Junior Spectrophotometer (19 × 105 mm. cuvette) and the amount of choline present was determined by reference to a standard curve. To determine bound choline, the lecithin was saponified with 0.5 *N* barium hydroxide (4 ml./25 mgm. lecithin) for two hours on the steam bath in a modified 50 ml. Erlenmeyer flask (8); the hydrolyzate, after acidification with glacial acetic acid and concentration on the steam bath to 2 ml., was extracted in the flask (8), with several portions of low boiling petroleum ether (30°–60°) to remove fatty acids. The free choline in the aqueous phase was estimated as described above.

Total chlorophyll was measured spectrophotometrically (652 mμ, Coleman Junior Spectrophotometer) on 85% acetone extracts (6) of 0.5 ml. aliquots of the chloroplast suspensions. The instrument was calibrated for chlorophyll by reference to a standard 80% acetone extract of leaf pigments, whose total chlorophyll content was determined according to MacKinney (19, 2), using a Cary recording spectrophotometer (24).

Dry weight determinations were made on the enzyme preparations by bringing 0.5 ml. aliquots to constant weight in a desiccator over sodium hydroxide at room temperature and reduced pressure (10^{-2} mm.).

Activity Assay Procedure

The systems used to measure lecithinase activity contained 0.5 ml. of 1 *M* acetate buffer (pH 4.7 for sugar beet or spinach, 5.5 for cabbage, and 5.8 for carrot), 0.5 ml. of a 5% lecithin emulsion, a suitable aliquot of plastid suspension (containing 100 to 130 mgm. of chloroplasts or 60 to 80 mgm. of carrot root plastids), and water to a total volume of 5.0 ml. The components were brought to 25° and mixed in a glass-stoppered tube (18 × 110 mm.). At zero time the mixture was shaken vigorously for 10 sec. with 3 ml. of diethyl ether (ACS reagent grade) and kept at 25 ± 1° for an appropriate length of time (usually 10 min. for sugar beet or spinach, 15 min. for cabbage or carrot). To stop the reaction, 1 to 2 ml. of 1 *M* trichloracetic acid (TCA) solution was added. The mixture was then extracted in the tube with three 10 ml. portions of ether, each extract being carefully decanted* after

** The insoluble part of the mixture, after centrifugation, usually formed a firm disc, fixed at the water–ether interface, which allowed decantation of the ether phase without loss of aqueous phase. When the disc did not remain fixed, an adaptor with a 5 ml. sidearm to retain the aqueous phase was used.*

centrifugation. The extracted mixture was freed from dissolved or entrained ether by stirring at 45°–50°, and was filtered through Whatman No. 50 or No. 2 filter paper. The filter was washed with distilled water and the filtrate was made up to an appropriate volume (usually 15 ml.). Aliquots were taken for determination of free choline and total and inorganic phosphate. When choline alone was to be determined, the extracted mixture, after removal of ether, was centrifuged, the supernatant was decanted into a 15 ml. centrifuge tube, and the residue was washed with 4–5 ml. of water; choline was then determined in the combined aqueous supernatants (9–10 ml.).

In each experiment, total choline and phosphate available from substrate was checked by analysis of 0.5 ml. aliquots of the 5% lecithin emulsion used. The values obtained (3.70 to 3.86 mgm. choline, and 0.97 to 1.01 mgm. P) were used to calculate the results as percentages of the total choline or phosphate available. Organic phosphate was determined as the difference between total and inorganic phosphate.

Blanks (substrate omitted) were always run simultaneously to correct for inorganic phosphate, water soluble organic phosphate, and substances forming insoluble reineckates, all of which are present in the enzyme preparations (see Table I). No free choline or phosphate was liberated when substrate was incubated with buffer in the absence of plastids or in the presence of boiled plastid suspensions. Known amounts of choline or inorganic phosphate added to buffered chloroplast suspensions were recovered quantitatively (Table I) after incubation and processing as described above.

TABLE I

RECOVERY OF CHOLINE AND PHOSPHATE ADDED TO BUFFERED SPINACH CHLOROPLAST SUSPENSIONS

Mixtures contained 76 mgm. dry weight of spinach chloroplasts, varying aliquots of choline chloride solution (0.0082 *M*), or potassium dihydrogen phosphate solution (0.00646 *M*), acetate buffer (pH 4.7), and water to total volume of 5.0 ml.; incubation with 10 ml. of ether, 10 min. for choline recovery, and 30 min. for phosphate recovery

Choline added, mgm.	Choline recovered[1]		P added, mgm.	P recovered[2]	
	Mgm.	%		Mgm.	%
0.496	0.51	103	0.100	0.099	99
0.992	1.03	104	0.200	0.203	102
1.49	1.49	100	0.300	0.291	97
1.98	2.00	101	0.400	0.390	98
2.48	2.49	100			
2.98	2.98	100			

[1] *Corrected for blank value of 0.22 mgm. of choline.*
[2] *Corrected for blank value of 0.201 mgm. of inorganic P.*

Experimental and Results

Intracellular Location of Lecithinase Activity

The choline-liberating activities of whole extracts of spinach, sugar beet, and cabbage leaves and of carrot root, and of their plastid and cell sap – cytoplasm fractions are given in Table II. In each species, the activity of the whole extract was found to be associated with the plastid fraction, while the cell sap – cytoplasm fraction showed negligible activity. Since the activity of the whole extract was always much lower than that of the corresponding plastid fraction the cell sap – cytoplasm appeared to be inhibitory. With spinach, this inhibition was especially marked and was further demonstrated by the great reduction in activity of the chloroplast fraction produced when it was diluted with the cell sap – cytoplasm fraction.

TABLE II

CHOLINE-LIBERATING ACTIVITY OF WHOLE EXTRACTS AND OF THEIR CELL SAP – CYTOPLASM AND PLASTID FRACTIONS

Reaction mixtures contained either whole extract (4.0 ml.), cell sap – cytoplasm fraction (4.0) or plastid fraction (to give same chlorophyll content and weight of plastid material as whole extract), buffer, substrate, and water, as in text; incubation with 1–3 ml. ether

Enzyme material		Dry wt., mgm./aliquot	Chlorophyll, mgm./aliquot	pH	Incubation time, min.	% Choline liberated
Sugar beet leaf	Whole extract	184	3.64	4.8	15	61
	Cell sap – cytoplasm	129	0.28			9
	Plastids (1.18 ml.)	47.5	3.66			80
Spinach leaf	Whole extract	127	1.86	4.8	25	32
	Cell sap – cytoplasm	105	0.10			3
	Plastids (0.52 ml.)	22.4	1.75			54
	Plastids + cell sap – cytoplasm*	114	1.84			25
Cabbage leaf	Whole extract	185	1.92	4.8	25	29
	Cell sap – cytoplasm	140	0.00			0
	Plastids (1.0 ml.)	41.6	1.84			44
Carrot root	Whole extract	178	—	5.8	30	32
	Cell sap – cytoplasm	163	—			4
	Plastids (0.62 ml.)	15.0	—			38

* *Plastids (0.52 ml.) diluted with cell sap – cytoplasm (3.48 ml.), instead of water.*

Activation by Diethyl Ether

Evidence for the activating effect of diethyl ether on the rate of choline liberation by spinach chloroplasts has been presented previously (16). The same effect has now also been observed with sugar beet and cabbage chloroplasts and with carrot root chromoplasts (Table III).

The activating effect of ether was found to depend on the concentration of ether in the system, as shown in Fig. 1. The rate of choline-liberation with

TABLE III

ACTIVATION OF CHOLINE-LIBERATION BY DIETHYL ETHER

Mixtures contained indicated amounts of plastids; other components as in text; incubation with or without 3 ml. of ether

Plastids			Incubation time, min.	% Choline liberated	
Source	Dry wt., mgm.	Chlorophyll, mgm.		With ether	Without ether
Sugar beet	80.4	6.20	15	84	1
Spinach*	78.0	4.92	60	69	< 1
Cabbage	107	4.45	60	87	0
Carrot root	50.5	—	60	80	0

** With and without ether, liberation of inorganic phosphate was 32% and 0.5% respectively; and of water-soluble organic phosphate, 8.3% and 1% respectively.*

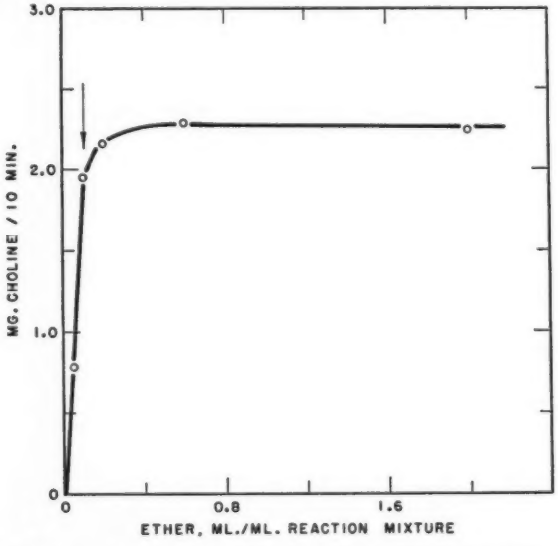

FIG. 1. Dependence of the rate of choline-liberation on the concentration of diethyl ether. Arrow indicates solubility of ether in water at 20° C. Reaction mixtures contained 79 mgm. dry weight of spinach chloroplasts; other components as in text.

spinach chloroplasts increased rapidly with increasing concentration of ether to a maximum and constant rate at saturation. The amount of ether subsequently used in the routine activity assay procedure was always in excess of saturation.

Although diethyl ether stimulated the enzymatic breakdown of lecithin, it also had an inactivating effect on the enzyme itself. For example, when a spinach chloroplast suspension was shaken with ether, in absence of substrate, the activity was found to decrease at the rate of about one per cent per minute over a 30 min. period. Such a rate of inactivation is however negligible compared to the initial rate of choline liberation in the presence of ether.

pH Dependence

Variation in the rate of choline-liberation with pH, for each of the plastid preparations studied, is shown in Fig. 2. For spinach and sugar beet, maximum activity was sharply defined at pH 4.7–4.8. For cabbage and carrot root, maximum activity was between pH 5 and 6, as previously reported for carrot root (11, 1) and cabbage leaf (12) extracts.

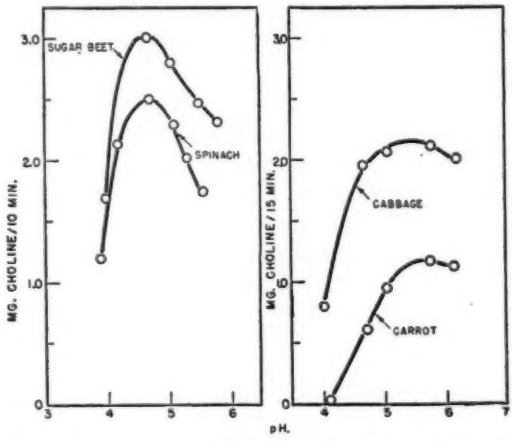

FIG. 2. Dependence of the rate of choline-liberation on pH. Reaction mixtures contained 80 mgm., 81 mgm., 84 mgm., or 48 mgm. of sugar beet, spinach, cabbage, or carrot plastids respectively; 0.5 ml. of 1 *M* acetate buffer (pH measured in presence of plastids); other components as in text; observed 30 min. liberations for carrot were converted to 15 min. liberations by multiplying by 0.5.

Influence of Plastid Concentration

For a fixed substrate concentration (0.5%), the rates of choline-liberation with all four plastid preparations were found to depend on the plastid concentration as shown in Fig. 3. The rates increased linearly at low plastid concentrations but fell off to maximum and constant values at higher concentrations. For the three choloroplast preparations, maximum rates were obtained at plastid concentrations greater than 20–24 mgm. per ml. of reaction mixture, while with the carrot chromoplasts the maximum was approached at concentrations greater than 12 mgm. per ml. Taking into account the difference in incubation times, Fig. 3 shows that sugar beet chloroplasts have the highest enzymatic activity, followed in decreasing order by spinach, cabbage, and carrot plastids.

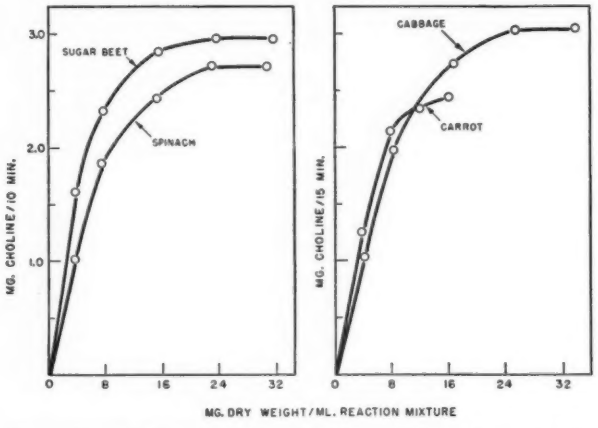

FIG. 3. Dependence of the rate of choline-liberation on plastid concentration. Plastid suspensions had the following chlorophyll and dry weight contents (mgm./ml.), respectively: spinach, 2.86, 38.4; sugar beet, 2.40, 39.6; cabbage, 1.78, 42.8; and carrot, —, 20.2. Reaction mixtures contained varying aliquots of these suspensions; other components as in text.

Influence of Substrate Concentration

With the chloroplast concentration fixed at a high value, the rate of choline liberation by spinach chloroplasts was found to depend on substrate concentration as shown in Fig. 4. An apparent Michaelis-Menten constant of

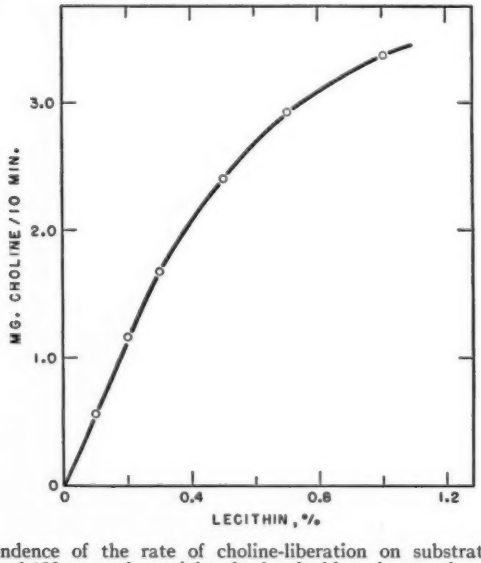

FIG. 4. Dependence of the rate of choline-liberation on substrate concentration. Mixtures contained 120 mgm. dry weight of spinach chloroplasts and varying amounts of a 5% lecithin emulsion; other components as in text.

$1.3 \times 10^{-2} M$ (taking 760 as the molecular weight of lecithin) could be calculated from the data, using the Lineweaver–Burk relation (18).

Influence of Incubation Temperature

Liberation of choline in 10 min. from lecithin by spinach chloroplasts at an incubation temperature of 35° was found to be 18% lower than that at 25°. Thermal inactivation of the enzyme at 35° was however negligible (see Fig. 5). Hanahan and Chaikoff (11, 12) have observed a similar effect of incubation temperature on the activity of the carrot root and cabbage leaf enzyme systems. They have suggested that this temperature effect might be due to the fact that the phospholipid emulsion is more easily broken at the higher temperature. However, from the present results, it is more probable that the lowered rate at 35° is due to decreased adsorption of the lecithin on the chloroplasts, together with increased rate of inactivation of enzyme by diethyl ether.

An incubation temperature of 25° has been used routinely throughout this work.

Thermal Inactivation

The effect of 10 min. heating at various temperatures on the activity of a spinach chloroplast suspension is shown in Fig. 5. The enzyme is relatively

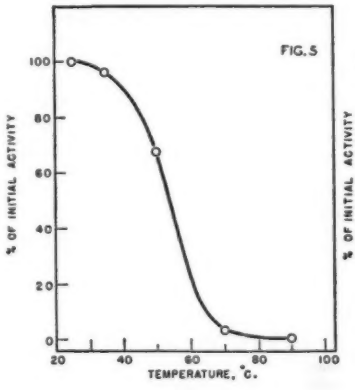

FIG. 5

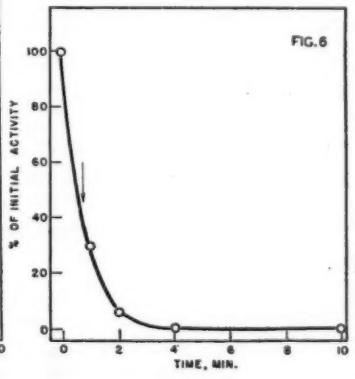

FIG. 6

FIG. 5. Thermal inactivation. Aliquots (2.5 ml.) of spinach chloroplast suspension (2.62 mgm. chlorophyll and 35 mgm. dry weight/ml.) heated at various temperatures for 10 min., immediately cooled to 25°, and activities measured. Initial activity, 2.22 mgm. choline/10 min.

FIG. 6. Rate of thermal inactivation at 70°. Aliquots (4.0 ml.) of whole extract of cabbage leaves (0.54 mgm. chlorophyll and 39.0 mgm. dry weight/ml.) heated at 70° for various times, immediately cooled to 25°, and activities measured. Initial activity, 1.36 mgm. choline/30 min. Arrow indicates coagulation time.

stable between 25° and 40° but is completely inactivated at 70°. Suspensions of sugar beet chloroplasts, cabbage chloroplasts, or carrot root plastids, as well as whole aqueous extracts of spinach or cabbage leaves were also found to

be completely inactivated after 10 min. at 70°. The much greater thermostability reported for aqueous extracts of carrot (11, 1) or cabbage (12) is difficult to reconcile with the present findings. However, the use of lecithin as substrate in this work instead of the lecithin–cephalin mixtures used in the earlier work may be responsible for these differences.

The rate of inactivation at 70° of a whole aqueous extract of cabbage leaves was investigated to determine whether activity still remains when cytoplasmic proteins and chloroplasts coagulate at this temperature (4, 3). Although the choline-liberating activity of the extract was completely lost after four minutes at 70°, at least 40% of the initial activity still remained when coagulation occurred (Fig. 6). The bearing of this finding on the explanation of the origin of the phosphatidic acids is discussed below.

Enzymatic Liberation of Inorganic and Water-soluble Organic Phosphate

The liberation of inorganic phosphate from phospholipids by plant enzymes has been reported previously (1, 14). The water-soluble products of lecithin hydrolysis by plastid suspensions from all four species were examined for the presence of phosphates as well as choline. The data given in Table IV show that under optimum conditions for choline-liberation, significant amounts of both inorganic and organic phosphate were liberated by all the preparations. Production of both inorganic and organic phosphate was much higher with spinach than with the other species. In absence of ether, when choline-liberation was negligible, liberation of inorganic and organic phosphate was also negligible with spinach chloroplasts (cf. Table III).

TABLE IV

WATER-SOLUBLE COMPOUNDS FORMED IN ENZYMATIC DEGRADATION OF LECITHIN

Mixtures contained indicated amounts of plastids; other components as in text; incubation, 60 min. for spinach and sugar beet, 100 min. for cabbage and carrot

Plastids			% Liberations of:		
Source	Dry wt., mgm.	Chlorophyll, mgm.	Choline	Inorganic P	Organic P
Spinach	108	8.43	84	53	9.4
Sugar beet	124	5.96	96	12	2.3
Cabbage	131	9.40	90	5.8	1.5
Carrot root	61.2	—	82	1.5	2.5

Fluoride Inhibition

Inhibition of phospholipid hydrolysis by sodium fluoride has been noted (1) with carrot press juice. The influence of sodium fluoride on the enzymatic hydrolysis of lecithin by spinach chloroplasts was therefore studied. The results (Table V) showed that fluoride inhibits not only the liberation of

inorganic phosphate, but also that of choline and water-soluble organic phosphate. These inhibitions were found to be reversed by an equivalent amount of calcium-ion, although it alone had little or no effect on the liberations.

TABLE V

FLUORIDE INHIBITION

Mixtures contained 72 mgm. dry wt. of spinach chloroplasts, varying amounts of 0.03 *M* sodium fluoride solution; other components as in text; incubation, 30 min. at 26°–27°

Final fluoride concentration, $\times 10^{-3} M$	% Inhibition of liberations of:*		
	Choline	Inorganic P	Water-soluble organic P
3.0	25	62	0
6.0	70	83	33
9.0	78	95	49
12.0	81	95	65

* *% Liberations of choline, inorganic P, and organic P in absence of fluoride were 71%, 7.5%, and 6.7% respectively.*

Discussion

The exact intracellular location of the enzymes studied here cannot be established with certainty until improved methods for the separation of cellular components have been developed. Nevertheless, the lecithin-hydrolyzing enzymes in plant extracts were found to be firmly associated with the plastid fractions and entirely absent from the cell sap – cytoplasm fractions, which were inhibitory.

These findings suggest that the apparent absence of lecithinase activity in spinach press juice reported by Acker *et al.* (1) may be related to the inhibitory action of the cell sap – cytoplasm and possibly to a low concentration of plastids in the press juice. The variations in activity reported for carrot press juice (1) may also be related to the plastid contents of the preparations.

Activation of a lecithin-hydrolyzing enzyme system by diethyl ether has been reported previously by Hanahan (10). He found that, on shaking an aqueous mixture of lecithinase A and lecithin with diethyl ether, the enzyme–substrate complex was extracted into the solvent where the reaction took place. This mechanism probably does not operate with the plastid lecithinase systems. The coalescence of the chloroplast and lecithin phases in ether-saturated systems (cf. 16) suggests that the reaction takes place in the insoluble plastid phase, and that ether promotes the adsorption of the substrate on the plastids. A detailed investigation of the mechanism of ether-activation of plastid lecithinase systems is in progress.

Chibnall and Channon (4) have isolated phosphatidic acid from ether extracts of cabbage leaf cytoplasm (a mixture of cytoplasmic proteins and

chloroplasts (3)), coagulated at 70°, but found little or no lecithin or cephalin. Subsequently, Hanahan and Chaikoff (12) found a heat-stable enzyme in cabbage leaf extracts, catalyzing the liberation of nitrogenous bases from glycerophosphatides, and suggested that the phosphatidic acid isolated by Chibnall and Channon might have arisen by enzymatic degradation of native glycerophosphatides during the isolation procedure. This interpretation receives further support from the results reported here. It has been shown, for example, that the choline-liberating activity of cabbage leaf extracts is associated entirely with the chloroplast fraction (Table II), and that 40% of this activity is still present at the time of coagulation of cytoplasmic proteins and chloroplasts at 70° (Fig. 6). Moreover, the enzymatic liberation of choline from lecithin is greatly stimulated by diethyl ether (Table III), while the rate of destruction of the enzyme in the presence of ether is negligible compared with the rapid rate of choline-liberation.

The present observations suggest that enzymatic degradation of native lecithin may occur during ether extraction of plant tissues in general, resulting in the formation of phosphatidic acid. The reported presence of phosphatidic acid or phosphatides with low N/P ratios in ether extracts of spinach leaves (5), runner bean leaves (15), *Hevea brasiliensis* latex (22), and of carrot root (11) supports this conclusion.

In addition to the choline-liberating enzyme, enzymes which catalyze the liberation of both inorganic and water-soluble organic phosphate from lecithin appear to be present in all the plastid preparations examined here (Table IV). The activity of the latter enzymes is especially pronounced in the spinach chloroplast fraction. Attempts are being made to identify the water-soluble organic phosphate present in the lecithin hydrolyzates. The course of hydrolysis of lecithin by the plastid fractions is also being studied to determine the sequence of reactions leading to the liberation of inorganic and water-soluble organic phosphate.

Acknowledgment

The author is indebted to Dr. P. R. Gorham for stimulating discussions and criticism during the course of this work. The technical assistance of Miss Mary Margaret Murray is gratefully acknowledged.

References

1. ACKER, L., DIEMAIR, W., and JÄGER, R. Biochem. Z. 322 : 471. 1952.
2. ARNON, D. I. Plant Physiol. 24 : 1. 1949.
3. CHIBNALL, A. C. Protein metabolism in the plant. Yale University Press, New Haven. 1939. p. 284.
4. CHIBNALL, A. C. and CHANNON, H. J. Biochem. J. (London), 21 : 225, 233, 1112. 1927.
5. CHIBNALL, A. C. and CHANNON, H. J. Biochem. J. (London), 23 : 182. 1929.
6. CLENDENNING, K. A. and GORHAM, P. G. Can. J. Research, C, 28 : 78. 1950.
7. DUCET, G. Ann. agron. 19 : 184. 1949.
8. ENTENMAN, C., TAUROG, A., and CHAIKOFF, I. L. J. Biol. Chem. 155 : 13. 1944.
9. GLICK, D. J. Biol. Chem. 156 : 643. 1944.
10. HANAHAN, D. J. J. Biol. Chem. 195 : 199. 1952.
11. HANAHAN, D. J. and CHAIKOFF, I. L. J. Biol. Chem. 168 : 233. 1947; 169 : 699. 1947.

12. HANAHAN, D. J. and CHAIKOFF, I. L. J. Biol. Chem. 172 : 191. 1948.
13. HANAHAN, D. J., TURNER, M. B., and JAYKO, M. E. J. Biol. Chem. 192 : 623. 1951.
14. HOLDEN, M. Biochem. J. (London), 51 : 433. 1952.
15. JORDAN, R. C. and CHIBNALL, A. C. Ann. Botany, 47 : 163. 1933.
16. KATES, M. Nature, 172 : 814. 1953.
17. KING, E. J. Biochem. J. (London), 26 : 292. 1932.
18. LINEWEAVER, H. and BURK, D. J. Am. Chem. Soc. 56 : 658. 1934.
19. MACKINNEY, G. J. Biol. Chem. 140 : 315. 1941.
20. ROSE, W. G. Food Technol. 4 : 230. 1950.
21. SMITH, R. A. Biochem. J. (London), 56 : 240. 1954.
22. TRISTRAM, G. R. Biochem. J. (London), 36 : 400. 1942.
23. WEIER, T. E. and STOCKING, C. R. Am. J. Botany, 39 : 720. 1952.
24. WOLKEN, J. J. and SCHWERTZ, F. A. J. Gen. Physiol. 37 : 111. 1953.
25. YASUDA, M. J. Biol. Chem. 94 : 401. 1931-32.

DESOXYRIBONUCLEIC ACID CONTENT AND CELL DENSITY IN BRAIN AND HUMAN BRAIN TUMORS[1]

By Irving H. Heller[2] and K. A. C. Elliott

Abstract

The desoxyribonucleic acid (DNA) content per nucleus and per unit weight of tissue have been determined chemically in normal cerebral cortex, cerebellar cortex, and corpus callosum of man, dog, and cat and in various human brain tumors. Nuclear densities have been calculated from these determinations. Corpus callosum contains approximately the same total number of cells as does cerebral cortex; cerebellar cortex contains several times this number. The nuclear density in tumors is usually higher than in cerebral cortex or corpus callosum. The amount of DNA per nucleus in primary brain tumors is considerably higher than in normal tissue. The average DNA per nucleus in the more primitive and malignant tumors appears to be higher than in the more differentiated tumors. Calculations indicate that the increase in the DNA per nucleus in brain tumors is more likely to be due to polyploidy than to increased mitotic activity.

Introduction

It is known that desoxyribonucleic acid (DNA) is confined to cell nuclei and that, with certain reservations, the average amount of DNA per nucleus in most normal tissues of a given species is constant (see e.g., 7, 12, 20, 22). By determining the DNA content per unit weight and per nucleus, an estimate of the number of nuclei per unit weight of a tissue can be obtained; this method has been applied by Davidson and Leslie (2) and others. The present paper gives the results of such determinations of DNA per nucleus and nuclear density in normal brain tissues and in human brain tumors. In all the primary brain tumors studied we have found that the amount of DNA per nucleus is considerably higher than in normal tissue.

In a subsequent paper the metabolic activities of normal brain tissues and brain tumors in relation to cell density will be compared.

Methods

The human material was excised during neurosurgical operations. Cats were decapitated with a large tree pruner, dogs were anesthetized and bled to death, and the brains were removed immediately. The tissue was placed in a dry closed jar and chilled in ice and then freed of blood and coarse membranes in a humid chamber. If determinations could not be done immediately the tissue was stored at about $-20°$ C. Normal cerebral or cerebellar cortex was sliced off with fine scissors or pinched off with fine forceps. An attempt was made to obtain the whole thickness of the cortex without white matter. The corpus callosum was dissected out. Tumors were

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separated from the adjacent brain. The tissue was ground in a mortar and duplicate samples were weighed out.

DNA per Nucleus

Nuclei were prepared and their number estimated by modifications* of the method of Cunningham, Griffin, and Luck (1).

The sample, 200–400 mgm. of tissue, was homogenized briefly with a Potter-Elvehjem homogenizer in cold 5% citric acid solution. The suspension was strained through several layers of gauze, made up to 30 to 40 ml. with the cold citric acid solution, and centrifuged for two to three minutes at low speed, about 40 × g, in a refrigerated centrifuge at 0° C. This brought down gross particles. The supernatant suspension was decanted and its volume measured and the number of nuclei contained in it was determined as follows: To a 1 ml. aliquot, 0.5 ml. of polychrome, methylene blue stain was added. A portion of this mixture was placed on a clinical hemacytometer chamber and all nuclei in the red cell squares were counted. The total number of nuclei in the supernatant suspension was calculated from the average of four separate counts.

The suspension was again centrifuged in the cold at rather low speed, about 100 × g, this time for 10–15 min. This brought down a large proportion of the nuclei leaving much extraneous material, including DNA from fragmented nuclei, in the supernatant fluid. The supernatant fluid was pipetted off and saved. The residue was taken up again with 5 ml. of cold citric acid solution and recentrifuged for 10 min. at 100 × g and 0° C. The supernatant fluid was added to the previous fluid. The combined volume of the supernatant fluids was measured and the number of nuclei present was determined as above and deducted from the previous count to give the number of nuclei remaining in the residue. The DNA content of this residue was then determined.

Determination of DNA

The isolation of DNA was carried out by the combination of the methods of Schmidt and Thannhauser (14) and Schneider (15, 17). The final determination was done by the ultraviolet absorption method of Logan, Mannell, and Rossiter (9). The latter authors showed that the colorimetric method of Dische (diphenylamine reaction) gives incorrectly high readings due to the presence of an interfering chromogenic substance in brain. We have confirmed this.

For the determination of DNA per unit weight of tissue, 100–200 mgm. of tissue were weighed and homogenized in about 5 ml. of cold 10% trichloracetic acid (TCA). For the determination of DNA per nucleus, the above-mentioned residue was taken up with cold TCA. Except for the final step, the whole process was carried out in a 15 mm. × 125 mm. test tube. Supernatant fluids were removed with a fine-pointed dropping pipette.

* *Most of the modifications were suggested by Dr. M. Enesco of the Department of Histology, McGill University.*

The suspension was centrifuged for 10 min. at 1000 × *g* at 0° C., the supernatant discarded, and the extraction with TCA repeated. The residue was washed with cold distilled water followed by cold 95% alcohol, and the residue was then heated carefully in a water bath at 70° C. for about three minutes with 5 ml. of a mixture of alcohol and ether (3 : 1). (A "Hengar granule" was used to prevent bumping and the tubes were covered with a blind funnel.) The suspension was centrifuged at 1000 × *g* for 10 min. at room temperature and the supernatant was pipetted off and discarded. The alcohol–ether extraction was repeated twice. These lipid extractions were omitted in the determinations on nuclei since the amount of tissue involved was small and the danger of loss was greater.

Potassium hydroxide, 1 ml. of 1 *N* solution per 100 mgm. original tissue used, was added to the residue and the tube was stoppered and kept at 37° for at least 15 hr. After being cooled in ice the DNA was precipitated by the addition of 0.2 ml. of cold 6 *N* hydrochloric acid and 1 ml. of cold 5% TCA per ml. of the potassium hydroxide solution previously used. After standing at 0–4° C. for 30 min. the DNA was centrifuged down and the supernatant discarded.

The residue was taken up with 1 ml. of water and 5 ml. of 5% TCA, and the tube was heated in the water bath at 85°–90° for 15 min., cooled, and centrifuged. The supernatant was transferred to a 10 ml. volumetric flask. The residue was washed with 2.5 ml. of the TCA and, after centrifuging, the supernatant was added to the flask and the volume made to 10 ml. with water.

Standard DNA solutions containing 25 to 250 μgm. DNA per ml. in water were prepared from desoxyribonucleic acid (Schwarz Laboratories) which contained 8.77% phosphorus. To 1 ml. of each standard and to a blank of 1 ml. water, 5 ml. of TCA were added and the procedure mentioned in the preceding paragraph was carried out at the same time as the unknowns. The absorption of the standards and of the unknowns was read at 268.5 mμ in 1 cm. quartz cuvettes with the Beckmann DU spectrophotometer. To come within the range of the standards the unknowns often had to be diluted; the unknown, diluted with water, was read against some of the blank similarly diluted.

Results

Normal Brain

Results obtained with normal brain tissues are shown in Table I. The average figures of 8.5 for the DNA content of dog cerebral cortex and 8.3 for corpus callosum, expressed as mgm. DNA–phosphorus per 100 gm. of tissue, were somewhat higher than those found by the same method by Logan *et al.* (9), whose average figures were 5.3 for cerebral cortex and 6.3 for white matter. The differences may be partly due to differences in the brain areas sampled. No previous figures for DNA per nucleus in brain have been reported. The figures for DNA per nucleus here reported, expressed as picograms (gm. × 10^{-12}) of DNA, are comparable to the value of 6.9 for human leucocytes obtained by Metais and Mandel (11) and to figures mostly

TABLE I

NORMAL BRAIN TISSUES

DNA per nucleus, total DNA content, and derived nuclear count

Tissue*	DNA† per nucleus, pgm.‡	DNA† per mgm. tissue, μgm.	Nuclei per cu. mm., thousands§
Human			
Cerebral cortex (parietal)	7.7, 8.1	0.91, 0.95	131
Cerebellar cortex	5.3, 7.3	5.9 , 6.2	852
Average	*7.1*		
Dog			
Cerebral cortex (central areas)	5.5, 7.8	0.92, 1.07	153
Cerebral cortex (central areas)		0.89, 0.98	144
Cerebellar cortex	5.9, 6.6	4.1 , 3.1	555
Cerebellar cortex		3.6 , 3.9	578
Cerebellar cortex		3.6 , 3.8	570
Corpus callosum		0.70, 0.96	128
Corpus callosum		0.93, 1.03	151
Corpus callosum		1.00, 1.04	157
Average	*6.5*		
Cat			
Cerebral cortex (frontal poles)	6.8, 7.9	1.00, 1.05	145
Cerebral cortex (mixed areas)	7.9, 8.1	0.63, 0.69	93
Cerebral cortex (mixed areas)		1.00, 1.02	142
Cerebral cortex (mixed areas)		0.93, 0.96	133
Cerebellar cortex	5.6, 6.5	5.0 , 5.9	768
Cerebellar cortex		5.3 , 5.8	782
Cerebellar cortex		6.0 , 6.4	874
Corpus callosum		0.89, 0.94	129
Corpus callosum		0.93, 1.09	142
Average	*7.1*		

* *The cerebral and cerebellar cortical samples probably included all architectonic layers in approximately their normal proportion.*

† *DNA–phosphorus may be estimated by multiplying the figures given by 0.0877.*

‡ *One picogram* $= 10^{-12}$ *gm.*

§ *Calculated from the mean values for DNA per mgm. and the average figure for DNA per nucleus in the same species, taking the density of the tissue as unity.*

around 5–7.5 obtained by a number of authors for various mammalian tissues and summarized by Davidson and Leslie (3). The figures shown in Table I for DNA per nucleus are mostly lower for cerebellar cortex than for cerebral cortex. This may be due to inaccuracy of determinations rather than a real difference; the figures for cerebellar cortex may be the more reliable because nuclear counting was more accurate in suspensions from this tissue.

The present method has not, apparently, been used previously to estimate cell densities in brain. By direct histological cell counting Tower and Elliott (21) tentatively estimated the average neuron density in dog and cat cerebral cortex to be about 24,500 and 30,800 per cu. mm. respectively. By an indirect method consisting of measurement of the total volume of cortex, the percentage of the volume occupied by neurons and the average size of the neurons, Shariff (18) has estimated that there are 29,000, 18,200, and 97,200 neurons per cu. mm. in human eulaminar, agranular, and konio cortex respectively. Our sample was eulaminar. Assuming that there are about five times* as many glial cells as neurons in the cerebral cortex, the total number of cells should be about six times the number of neurons. Application of this factor gives figures for total cell density of the same order as those now found by the chemical estimate of nuclear density.

The chemical method may be more accurate and less laborious than the histological methods. It could be useful in relating various metabolic activities to cell density and in estimating total cell densities in different brain regions in various animals and in human subjects of varying mental condition or intelligence. The chemical method, however, does not differentiate between neurons and glial cells and histological estimates of neuron/total cell ratios would still be needed if estimates of neuron densities are required in brain tissues.

Table I shows the results obtained with three different brain structures of cat, dog, and man. The nuclear density of cerebellar cortex is found, in accordance with histological observations, to be several times as great as that of cerebral cortex. It is of interest to note that the corpus callosum contains approximately as many nuclei as does cerebral cortex in spite of the absence of nerve cell bodies and the large proportion of myelin. Logan *et al.* (9) have previously found the DNA content of white matter to be slightly higher than that of cortical gray matter in the dog.

Brain Tumors

Results obtained with brain tumor tissues are shown in Table II. Some of the duplicate determinations show poor agreement; this may be due to incomplete mixing before sampling these highly nonhomogeneous tissues. In all the tumors arising from ectodermal elements and in a meningioma, the DNA per nucleus was found to be considerably greater than in normal human tissue. Though variability in the results prevents establishment of definite correlations, it seems that tumors of more primitive and more malignant type (medulloblastomas, astroblastomas, and "malignant gliomas") often have higher DNA per nucleus values than the more differentiated tumors (oligodendroglioma and astrocytomas) which in general are clinically and pathologically more benign.† A bronchiogenic carcinoma metastasis to the brain gave values only slightly higher than those for normal tissue.

* *This factor was suggested by Dr. Jerszy Olszewski of this Institute.*

† *The averages for the two groups, 12.2 and 9.9 picograms per nucleus, are significantly different.* ($P < 0.05$).

TABLE II

HUMAN BRAIN TUMORS

DNA per nucleus, total DNA content, and derived nuclear count

Tissue	DNA per nucleus, pgm.	DNA per mgm. tissue, μgm.	Nuclei per cu. mm., thousands*
Medulloblastoma	13.2, 13.6	4.4, 4.0	313
Medulloblastoma	9.1, 11.0	11.7	1116
Astroblastoma	12.2, 12.9	4.6, 4.3	354
Malignant glioma†	12.2	4.9	402
Malignant glioma†	14.2, 17.1	1.3, 1.8	99
Glioblastoma (solid)	8.7, 13.5	1.9, 1.9	171
Glioblastoma (bloody)	7.6, 13.7	7.3, 8.4	738
Astrocytoma, piloid	8.0, 11.1	1.4, 1.9	173
Astrocytoma, piloid	9.9, 11.6	3.8, 5.2	418
Astrocytoma, piloid		2.1	206‡
Astrocytoma, piloid (calcified)	9.4, 10.3	2.5, 2.7	264
Astrocytoma diffusum	9.3, 10.6	0.87	88
Oligodendroglioma (calcified)	8.2, 10.3	2.6, 2.9	298
Meningioma	9.9, 10.7	5.0, 5.3	500
Bronchiogenic carcinoma metastasis	7.0, 8.3	4.5, 4.6	595

* *Calculated from mean values of DNA per nucleus and DNA per mgm. obtained from the individual tumor. Density taken as unity.*

† *Malignant glioma of uncertain type with a great deal of necrosis.*

‡ *Calculated from the average DNA per nucleus for astrocytomas.*

There appear to have been no previous determinations of DNA in brain tumors. By chemical methods, Price *et al.* (13) found increased DNA in the nuclear fraction of liver tumors induced by aminoazo dyes in rats but concluded that this was mainly due to an increased number of nuclei. Cunningham *et al.* (1) found a small increase per nucleus in rat liver tumors induced by an aminoazo dye but not by acetylaminofluorene. They attributed the increase to increased polyploidy. (It is known that a number of polyploid cells occur in normal liver.) By microphotometric determinations on Feulgen-stained preparations, Mark and Ris (10) found that the nuclei of cells of similar size and shape (small spherical) in normal liver and in hepatomas and cholangiomas contained equal amounts of DNA. Much higher values than in nuclei from normal mammalian tissues have been found in cells of the Erlich but not the DBA mouse ascites tumor by Leuchtenberger *et al.* (6) using chemical and spectrophotometric methods, and much higher values than in normal fowl nuclei have been found, by a chemical method, in fowl sarcoma by Davidson and McIndoe (see (3)). By the microphotometric method, Stowell (19) found higher than normal average amounts of DNA per nucleus in various human

tumors but Metais and Mandel (11) found no significant elevation of the DNA content of white blood cells from patients with leukemia or Hodgkin's disease. By chemical methods, Davidson *et al.* (4) found a wide range of DNA per nucleus in normal human bone marrow but no significant average difference in leukemic patients. While the present work was in progress, the results of a detailed study on the DNA per nucleus in normal human tissues and various human tumors (not including brain and brain tumors) were published by Leuchtenberger, Leuchtenberger, and Davis (7). Microspectrophotometric analysis of individual Feulgen-stained nuclei showed that all normal human tissues contain cells which vary somewhat individually but contain a constant mean DNA content per nucleus; in addition, cells containing multiple amounts of DNA were found in liver, urinary bladder, and kidney. In many types of precancerous and malignant tissues the DNA per nucleus was more variable and often, but not always, considerably higher than the normal mean. Leuchtenberger *et al.* considered that the increased DNA per nucleus was simply a function of increased mitosis. Their measurements were made only on cells in interphase but they pointed out that the build-up of DNA occurs at an early stage in mitosis before structural changes can be visualized.

It is difficult to believe that increased mitotic activity can be the full explanation for the elevated DNA per nucleus, at least in brain tumors. If every nucleus contains either the basic amount or n times the basic amount of DNA, it can be shown (see appendix) that the percentage, P, of nuclei containing n times the basic DNA content is given by $P = 100[(R-1)/(n-1)]$ where R is the ratio of the average DNA per nucleus in the abnormal tissue to that in the normal. The more active group of brain tumors studied contained an average of 12.2 picograms of DNA per nucleus while the content in normal cells was 7.1. If these tumors contained only cells with the normal and twice the normal resting amount of DNA, the above equation would indicate that about 70 per cent of the cells have twice the resting content. If there are cells in the initial stages of mitosis which are building up their DNA but have not yet reached double the resting DNA content, that is, n averages less than 2, then the above equation would indicate that even more than 70 per cent of the cells must be approaching mitosis. It seems quite improbable that such a high proportion of cells are in some phase of mitosis at any one moment. It is more probable that polyploidy accounts to a considerable extent for the high average figure for DNA. Only 24% of the nuclei need to be octaploid, for example, to give the increased average DNA per nucleus found. It is possible that polyploidy may occur in some noncancerous brain cells since Leuchtenberger *et al.* (7) and others have found cells with high DNA content in other normal human tissues. The *increased* occurrence of polyploidy in tumors could be secondary to some aspect of the abnormal habit of these tissues such as their increased rate of growth. But there is the possibility that

increased polyploidy is an essential feature of the development of the malignancy. (There is also the possibility that changes occur which result in polyteny, i.e., increased DNA associated with normal numbers of chromosomes.) The variability of the chromosome structure and the occurrence of aneuploid, either hyperdiploid or hypotetraploid, nuclei in tumor cells, particularly ascites tumors, have been described by Levan and Hauschke (8). It may be mentioned that, during the counting of nuclei in the present work, large misshapen nuclei which may have been polyploid were seen to be scattered among normal-appearing nuclei in most of the tumors.

Davidson and Waymouth (5), Schneider (16), and others have found increased amounts of DNA in tumors in experimental animals. The DNA content per unit weight of most of the brain tumors studied (Table II, column 3) is higher than that of cerebral cortex, corpus callosum, and presumably most other parts of the brain except cerebellar cortex. This is accounted for by their usually greater cellularity and the increased DNA per nucleus. The histologically well-known variability in cellularity of brain tumors is illustrated in the fourth column of Table II which shows widely varying numbers of nuclei per unit weight among tumors of similar type.

Appendix

Calculation of Percentage of Nuclei with Elevated DNA Content

Assume that, in the normal tissue, all nuclei contain an equal basic amount of DNA and that, in the tissue which has an elevated average DNA/nucleus, the nuclei contain either the basic amount of DNA or a single multiple, n, of this basic amount.

Let D_N = basic DNA/nucleus,

and let D_M = average DNA/nucleus in the tissue with elevated DNA/nucleus.

In unit volume of the tissue with the elevated average DNA per nucleus

let N = number of nuclei containing the basic DNA/nucleus,

and let M = number of nuclei containing n times the basic DNA/nucleus.

Let R = the ratio D_M/D_N.

$$\text{Then } D_M = \frac{(D_N \times N) + (nD_N \times M)}{N + M} = \frac{D_N\,(N + nM)}{N + M},$$

$$R = \frac{D_N\,(N + nM)}{N + M} \times \frac{1}{D_N} = \frac{N + nM}{N + M},$$

$$\text{whence } M = \frac{(1 - R)\,N}{R - n}.$$

Let P = the percentage of cells which contain the elevated DNA/nucleus

$$= \frac{100\ M}{N + M}.$$

Substituting the above formula for M and rearranging,

$$P = \frac{100\ (R - 1)}{n - 1}.$$

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References

1. Cunningham, L., Griffith, A. C., and Luck, J. M. J. Gen. Physiol. 34 : 59. 1950.
2. Davidson, J. N. and Leslie, I. Nature, 165 : 49. 1950.
3. Davidson, J. N. and Leslie, I. Cancer Research, 10 : 587. 1950.
4. Davidson, J. N., Leslie, I., and White, J. C. J. Pathol. Bacteriol. 63 : 471. 1951.
5. Davidson, J. N. and Waymouth, C. Biochem. J. (London), 38 : 379. 1944.
6. Leuchtenberger, C., Klein, E., and Klein, G. Cancer Research, 12 : 480. 1952.
7. Leuchtenberger, C., Leuchtenberger, R., and Davis, A. M. Am. J. Pathol. 30 : 65. 1954.
8. Levan, A. and Hauschke, T. S. J. Natl. Cancer Inst. 14 : 1. 1953.
9. Logan, J. E., Mannell, W. A., and Rossiter, R. J. Biochem. J. (London), 51 : 470, 480. 1952.
10. Mark, D. D. and Ris, H. Proc. Soc. Exptl. Biol. Med. 71 : 727. 1949.
11. Metais, P. and Mandel, P. Compt. rend. soc. biol. 144 : 277. 1951.
12. Mirsky, A. E. and Ris, H. Nature, 163 : 666. 1949.
13. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M. Cancer Research, 10 : 18. 1950.
14. Schmidt, G. and Thannhauser, S. J. J. Biol. Chem. 161 : 83. 1945.
15. Schneider, W. C. J. Biol. Chem. 161 : 293. 1945.
16. Schneider, W. C. Cancer Research, 5 : 717. 1945.
17. Schneider, W. C. J. Biol. Chem. 164 : 747. 1946.
18. Shariff, G. A. J. Comp. Neurol. 98 : 381. 1953.
19. Stowell, R. E. Cancer Research, 6 : 426. 1946.
20. Swift, H. H. Physiol. Zool. 23 : 169. 1950.
21. Tower, D. B. and Elliott, K. A. C. Am. J. Physiol. 168 : 747. 1952.
22. Vendrely, C. Bull. biol. France et Belg. 86 : 1. 1952.

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